THE ROLE OF GRAPE-DERIVED PROTEIN IN LIMITING RED WINE TANNIN

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Although they possess significant viticultural advantages, interspecific hybrid grapes (*Vitis* spp.) are reported to produce wine with lower condensed tannin (CT) concentrations than premium European wine varieties (*V. vinifera*). To elucidate the factors responsible, wines were produced from both red hybrid and *vinifera* cultivars under identical conditions in the Finger Lakes American Viticultural Area (AVA). Wine CT quantities varied across cultivars by up to 17 fold, while fruit CT differed by only up to 6 fold. CT in wines produced from *V. vinifera* grapes were an order of magnitude higher those in hybrid species (<50 mg/L), but lower than average CT values reported for US West Coast wines, 255 vs 544 mg/L. Further experiments in which cell wall material was incubated with CT indicated that CT binding to cell wall material may be of greater importance than grape CT for explaining wine CT variation. Cell wall characterization revealed that protein in flesh and, to a lesser extent, pectin in skin cell walls were correlated with CT binding (r^2=0.597 and r^2=0.255, respectively).

CT retention in finished wines was investigated by adding purified CT to native *Vitis*, hybrid and *vinifera* wines, leading to a precipitate with high nitrogen content. Proteomic analysis of the CT precipitate identified several classes of pathogenesis-related (PR) proteins. Protein concentration in juices and red wines were quantitated by SDS-PAGE and were highest in native *Vitis* spp. (juice= 706 mg/L, wine=296 mg/L), followed by interspecific hybrids (juice= 176 mg/L, wine=92 mg/L) and *Vitis vinifera* (juice = 146 mg/L, wine=16 mg/L). The fining of added CT by wine protein was modeled by the Freundlich equation (r^2=0.605). To evaluate the role of grape-derived proteins in limiting CT extractability, *V. vinifera* and interspecific hybrids from both hot and cool climates were vinified under controlled conditions. Final CT concentration in

wine was well modeled from initial grape tannin and juice protein concentrations using the Freundlich equation ($r^2 = 0.686$). In follow-up experiments, pre-fermentation removal of juice protein by bentonite increased wine CT, suggesting that this treatment may be a viable way to increase CT extractability.

BIOGRAPHICAL SKETCH

Lindsay Springer joined the lab of Dr. Gavin Sacks in the Department of Food Science at Cornell University in 2012. Her research focuses on factors that limit the extraction of CT from grapes into wine, and has been featured in publications such as the *Cornell Chronicle*, *Wines and Vines* Magazine, and *Chemical & Engineering News*. Lindsay's research efforts have been recognized by the American Chemical Society's Division of Agriculture Withycombe-Charalambous Graduate Research Award, multiple presentation awards from the American Society for Enology and Viticulture, and recognitions from the American Wine Society, the Juice Products Association, and Western NY Institute of Food Technologists.

Lindsay previously earned her Bachelor of Science degree in Biomedical Sciences in 2007, followed by M.S. degrees in Biomaterials and Pharmaceutical Sciences at the University at Buffalo (UB) in 2010. She is the primary inventor of a xerostomia relief mouth rinse, trade named Lubricity™, now licensed by UB to a local start-up company. Lindsay has been involved with the Cornell Center for Teaching Excellence through a Research as Teaching Fellowship, and plans to pursue a career in academia after defending her dissertation.

In loving memory of Alfred and Elizabeth Brennan

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CHAPTER 1

INTRODUCTION

Tannins are a class of secondary metabolites synthesized in the leaves, stems, and fruits in a wide array of plant species. As a functional definition, Bate-Smith and Swain (1962) deemed tannins "water soluble phenolic compounds having molecular weights between 500 and 3,000 (Da) and, besides giving the usual phenolic reactions, they have special properties such as the ability to precipitate alkaloids, gelatin, and other proteins."[1] More recent research has identified larger polyphenols and their metabolites (up to 20,000 Da) that can precipitate proteins, expanding the previous previsions of what qualifies as tannin (but with the questionable water solubility).[2] Plant polyphenols, typically those derived from oak (the root word 'tann' refers to oak in Celtic), have been used to fix animal hides into leather (tanning process) since 1500 BC.[3] The interaction between tannin and protein is well established, especially for proline rich proteins.[4] In addition to opening up the protein structure, the pyrrolidine face of proline residues interacts with hydrophobic aromatic rings of polyphenols through hydrophobic stacking.[5] Tannins presumably serve as an antiherbivory defense mechanism by preventing the digestion and absorption of dietary protein by ruminants,[6] in addition to their role as antioxidants in plants.[7]

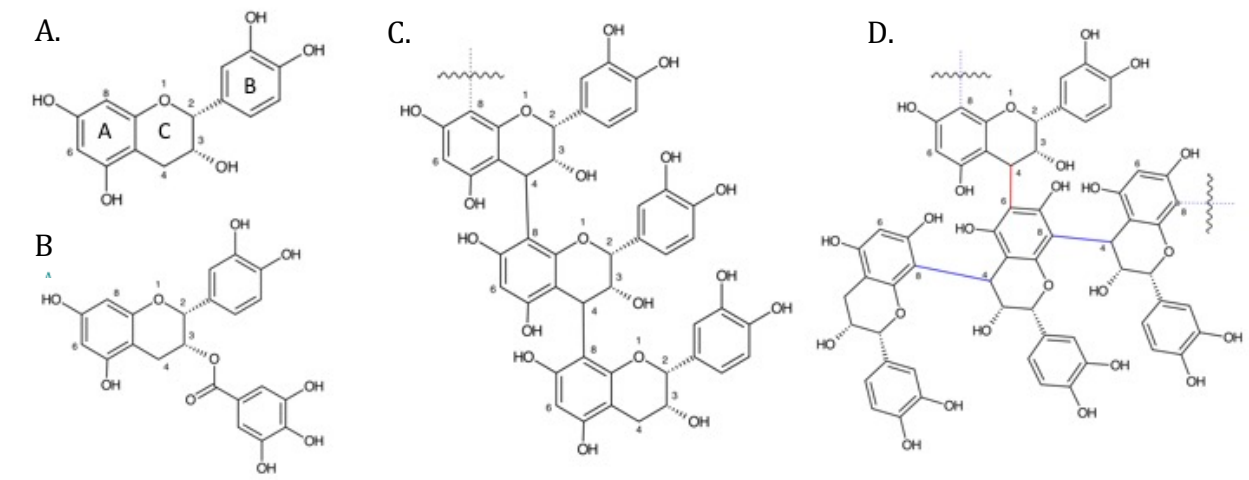
Condensed tannins (CTs), or proanthocyanidins, are a class of flavan-3-ol polymers exhibiting extraordinary structural diversity. Each flavan-3-ol building block contributes multiple hydroxyl groups to foster hydrogen bonding interactions, as well as aromatic rings that stabilize hydrophobic interactions, making CTs large, amphipathic molecules (Figure 1.1). CTs can differ in molecular size (number of flavan-3-ol subunits), flavan-3-ol composition and interflavan bond type, which will in turn govern the number and extent of interactions it can have

with other macromolecules, as determined by the amount and types of hydrogen bonding and hydrophobic interactions, as well as molecular confirmation.[5]

Condensed Tannin in Grapes

In grapes, CT is primarily located in the hypodermal cell layers of the skin and parenchyma layer of grape seeds, between the cuticle and hard seed coat.[8] Flavanoid biosynthesis, through assembly of flavan-3-ols, takes place via the phenylpropanoid pathway, on the cytosolic face of the rough endoplasmic reticulum from fruit set until veraison.[7, 9] It is hypothesized that flavan-3-ol monomers are then transported into vacuoles for polymerization,[10] however details surrounding polymerization still remain a mystery. At maturity, seeds are reported to contain up to 75%-96% of the solvent extractable CT in grapes, with the remainder residing in skins.[9, 11, 12]

Figure 1.1 CT Chemistry



A. The chemical structure of epicatechin
B. The chemical structure of epicatechin gallate
C. An example of a linear CT consisting of repeating epicatechin subunits polymerized via 4-8 interflavan bonds
D. An example of a branched CT chemical structure, incorporating both 4-8 (blue) and 6-8 (red) interflavan bonds

The average molecular size, or mean degree of polymerization (mDP, average number of flavan-3-ol subunits per CT molecule), and flavan-3-ol make-up of CT molecules differs by

grape tissue of origin. CT molecules derived from skins are much larger (up to ~85 mDP[13]) than those derived from seeds (mDP up to ~12[14]), and also differ in flavan-3-ol composition. CT derived from both skin and seed feature epicatechin as an extension unit (Figure 1.1 A) and its epimer, catechin, as a terminal or extension unit.[10] Unlike CT derived from seed, CTs from skin also contain the flavan-3-ol epigallocatechin. Epigallocatechin, a prodelphinidin, is simply epicatechin with a trihydroxylated A ring, increasing the potential number of hydrogen bonds over epicatechin. The shorter CT molecules derived from seeds contain epicatechin 3-O-gallate (shown in figure 1.1B), which do not typically appear in skin-derived CT molecules. The added gallate ester contributes 3 additional hydroxyl groups for hydrogen bonding, and additional aromatic ring to stabilize hydrophobic interactions.[15] Because of these extra components, gallated CT molecules derived from seeds have been shown to bind protein more efficiently than non-gallated molecules of a similar size (mDP).[16] CT molecules in grapes typically utilize a combination of 4-8 and/or 6-8 interflavan bonds which impart linear or branched conformations, respectively (Figure 1.1C & 1.1D). Branched CT configurations (6-8 bonds) impart less intramolecular mobility and thus reduces its ability to interact with other macromolecules, compared to the linear form.[17] Similarly, flexible, linear proteins will bind CT more efficiently than compact globular proteins.[4]

Condensed Tannin in Red Wines

The amount of CT in red wines is directly proportional to the tactile sensation of astringency, while CT condensation products with other wine components, such as anthocyanins, are related to other indicators of red wine quality (i.e. color stability).[11] CT elicits the sensation of astringency by inhibiting the functions of lubricatory proteins in the oral cavity, interacting directly with oral mucosa proteins, and/or triggering trigeminal receptors.[18, 19] Molecules with an

mDP>3 can bind to protein to elicit astringency, and as a result of this mechanism protein precipitation methods to quantify CT have been successfully applied to model red wine astringency intensity.[20, 21] In red wines, the primary structural diversity of CT imparted by subunits, molecular size, and linkage type is further complicated when secondary CT condensation products are formed. Anthocyanins, together with other electrophilic wine components (e.g. acetaldehyde), can further modify the CT molecule throughout the course of fermentation and during the wine aging process. CT modification reduces the capacity of the CT molecule to interact with proteins by shielding phenolic hydroxyl groups (thus reducing hydrogen bonding potential) and diminishing the polymer's flexibility. Consequently, polymeric pigment, the anthocyanin-CT adduct responsible for red wine color stability, is less astringent than equivalent amounts of unmodified CT.[22] The sub-qualities of red wine astringency, such as grippiness or velvet-like, are currently thought to relate to CT composition, or the complex secondary structures resulting from oxidation.[23]

Research has identified a positive correlation between the amount and molecular size of CT, and perceived red wine quality in wines produced from European wine grapes, *V. vinifera.* [24, 25] However, the understanding of factors responsible for CT extraction and retention is limited. The extraction of CT from grapes into the liquid fraction of the must (crushed and fermenting grapes) requires contact between the grape solids (skin, seeds) and the liquid phase. Juice, before fermentation, contains minimal quantities of CT. As alcoholic fermentation begins, skin CT extraction progresses as ethanol increases and skin tissues degrade. The concentration of skin CT typically plateaus just before the end of alcoholic fermentation.[26] The extraction of seed-derived CT experiences a lag phase as the outer seed coat hydrates, then proceeds at a linear rate until the wine is separated from the solids.[27] Therefore, in principle, the amount and type of CT

in red wines can be modified by manipulating contact time with grapes solids (e.g. extended maceration), and maceration conditions (e.g. mechanical agitation).[11] In a survey of commercial red *vinifera* wines from the US West coast and from Australia, variation in final wine CT quantities ranged from 30-1895 mg/L.[28] By comparison, variation in grape CT is only cited to range 2 to 4 fold.[29, 30] However, this variation in wine CT is not entirely within the winemaker's control, as the amount and extent of CT extraction during fermentation, and the effectiveness of cellar treatments to manipulate extraction, is difficult to predict and heavily dependent on variety. A poor correlation between CT in grape and final wine CT is well documented in the literature, while the variation in CT extraction across *vinifera* wine grape varieties is cited to range from 4.9-61%.[28] This is in contrast to other phenolic species, such as anthocyanins, that demonstrate excellent fruit to wine correlations using identical protocols.[31]

Because CT is critical to red wine quality, winemakers often add commercial CT preparations directly to their wines at different stages of winemaking in an effort to improve mouthfeel, color stability, and other sensory properties.[32] However, even after accounting for the low purity of these products (one published survey of commercial CT preparations reported purity levels ranging from 12-48%[33]), the amount of added CT retained is generally quite poor.[33-36] CT extraction and/or retention is i) typically much lower than 100% and ii) may vary by an order of magnitude even under identical vinification conditions (Table 1.1).

TABLE 1.1 Literature Summary- Percent of Condensed Tannin Recovered After Addition, or Extracted During Fermentation

Ref	Experimental Design	CT Extraction or Recovery
[33]	60-300mg/L commercial CT added to finished Merlot wine	ND-79%, depending on dose
[37, 38]	400 mg/L commercial CT added to Monastrell fermentation at crush	ND-<50%, depending on vintage
[35]	200 mg/L seed CT added to Shiraz pre- or post-fermentation	ND-<25%
[34]	400 mg/L commercial CT added to Corot noir and Maréchal Foch at crush	<20%
[39]	Added 0.5-2.0 g/L of seed CT to a low polyphenol port wine	2-5%
[36]	200 mg/L seed CT added to Cynthiana wine post fermentation	ND
[30]	CT measured in Pinot noir, Cabernet Sauvignon, Syrah fruit and wines	4.9- 61%, depending on variety

To date, two main hypotheses have been proposed to explain why CT extraction and retention are challenging to predict and why the amount of CT in grapes is a poor indicator of CT in wines. Given their interactions with other macromolecules, CT binding to structural cell wall carbohydrates via hydrogen bonding and hydrophobic interactions has been a prominent theory in the scientific literature.[40] Research on CT extraction from cider apples demonstrated CT had the highest affinity for pectin, with lesser affinity for cell wall carbohydrates xyloglucan, starch and cellulose.[41] The molecular flexibility of pectin has been proposed to allow the formation of hydrophobic pockets during grape berry ripening, increasing cell wall surface area for CT binding and modulating CT extraction into wine.[42, 43] Thus, changes in cell wall composition and structure during ripening, particularly those involving pectin, have been hypothesized to play a major role in CT extraction into wines.[44]

An alternate hypothesis is that certain grape components must be present to facilitate CT extraction. Specifically, anthocyanins have been proposed to enhance the solubility of CT via polymerization,[45] and this was first suggested to explain the observed differences in CT extraction between red and white wines. Other authors who observed higher CT in wines with higher anthocyanin concentrations propose that anthocyanins can compete with CT for binding sites on cell walls, driving more CT into wine.[46, 47] However, this hypothesis does not explain why wines produced from interspecific hybrid grapes, which contain large amounts of anthocyanins, have very poor CT extraction and retention in their respective wines.[34]

The Case of Interspecific Hybrid Grapes

Grape breeders have crossed native *Vitis* spp. with *vinifera* wine grapes to create new interspecific hybrid grape varieties (i.e. Maréchal Foch, Corot noir) with improved resistance to biotic and abiotic stresses in the vineyard. These rugged hybrid varieties are grown in nontraditional wine regions, such as the cool and humid Northeastern United States, setting up new variety-environment combinations, and consequently new challenges for winemakers. Although they possess viticultural advantages over *V.vinifera* species, red wine produced from interspecific hybrid varieties can exhibit undesirable traits, including a 'thin' mouthfeel due to a lack of CT. In the few published studies investigating interspecific hybrid varietals, wines produced from Corot noir and Maréchal Foch were reported to have <100mg/L of protein precipitable CT, while the average red wine produced from *V.vinifera* contains ~500 mg/L of CT.[28, 48, 49] In addition to exhibiting low CT extraction into wines, adding CT directly to interspecific hybrid fermentations yields exceptionally low recovery.[34, 36]

PROJECT OVERVIEW

Since winemaking efforts to improve mouthfeel and CT quantities in interspecific hybrid wines have fallen short of success, and the variation in CT extraction from *vinifera* varieties is still not understood, the biochemical determinants of CT extraction and retention in red wine is the focus of this dissertation research.

For this work, 44 of 51 vinified grapes samples (native *Vitis* spp., hybrid *Vitis* spp., and *V.vinifera*) were sourced from the Finger Lakes AVA of New York (NY). The NY wine industry boasts a 4.8 billion dollar impact on the state's economy, and the Finger Lakes region continues to be recognized for its world class Riesling. But despite the region's success with Riesling and other white *vinifera,* red wines from this area are not on the premium level set by those on the west coast (e.g. CA). Given the array of red interspecific hybrid and *vinifera* grape varieties grown in the region, and its red wine quality challenges, the Finger Lakes was a unique opportunity for a project investigating CT extraction and retention.

In the first chapter of experiments, two main hypotheses were tested:

1. *V.vinifera* varieties from the Finger Lakes contain higher quantities of CT in their skins, seeds, and finished wines as compared to interspecific hybrid varieties.
2. Cell wall materials derived from interspecific hybrid grapes are able to bind significantly more CTs than those derived from *V.vinifera*.

Understanding the relationship of CT quantities in fruit, wine, and the ability of grape-derived materials to bind CT was an important first step in understanding the documented differences hybrid and *vinifera* wines. Both pectin and protein are quantified to explore relationships between binding affinity and chemical composition. A survey of commercial wines also contributes an important validation of the experimental results obtained for CT quantity,

demonstrating that the employed small scale fermentation set-up is relevant to commercial practice.

In the second chapter, PR proteins are identified in precipitates derived from finished red wines after adding CT, setting up two new hypotheses to investigate:

3. Juices and wines derived from interspecific hybrid grapes contain more PR proteins than those derived from *V.vinifera* grapes.
4. Residual protein in red wines will lead to losses of added CT via precipitation.

A quantitative and qualitative assessment of juice and wine proteins are documented for native Vitis, interspecific hybrid, and *V.vinifera* varieties, and their ability to fine (adsorb) CT is evaluated using a Freundlich model.

In the third and final series of experiments, grapes from both NY and California (CA) are employed to assess the effects of soluble juice protein on CT extraction during fermentation, and approach our hypothesis using grapes from a vastly different growing region. The following hypotheses are tested:

5. Soluble grape-derived protein in juices will be a significant predictor in modeling final wine CT across all grape varieties, superior to that of solely CT in fruit
6. Reducing the amount of protein in juice before fermentation begins will increase the amount of CT extracted into wine.

The Freundlich model is revisited to describe the CT fining phenomena set by protein and demonstrate a fundamental concept in CT extraction that encompasses data derived from both NY and CA sourced fruit. To confirm the role of protein, further experiments to remove juice protein and enhance CT extraction are shown. A discussion of the challenges of grape derived protein quantification and future work concludes this project

CHAPTER 2

PROTEIN-PRECIPITABLE TANNIN IN WINES FROM *VITIS VINIFERA* AND INTERSPECIFIC HYBRID GRAPES (*VITIS* SPP.): DIFFERENCES IN CONCENTRATION, EXTRACTABILITY, AND CELL WALL BINDING[a]

INTRODUCTION

European winegrape (*Vitis vinifera,* abbreviated as *vinifera*) is the major grape species used for wine production, representing 95% of the world winegrape market by tonnage.[50] Because it evolved in a Mediterranean climate, *vinifera* grapes can be challenging to grow profitably in cool and humid climates due to their susceptibility to disease pressure and poor cold hardiness.[51] Consequently, interspecific hybrid grapes (*Vitis* spp.) with *vinifera* and wild American *Vitis* species (e.g. *Vitis rupestris, Vitis riparia*) parentage have been developed, as these are suited for a wider range of climates and disease pressures. In response to the introduction of the phylloxera root louse to Europe during the late 19th and early 20th centuries, French-American hybrids were bred by directly crossing *vinifera* with wild American *Vitis*. These cultivars have largely been replaced in most European vineyards with *vinifera* grafted to resistant rootstocks, but continue to be cultivated for wine production elsewhere, particularly cool- and humid- regions of North America.[51] Newer cultivars of hybrid grapes with a more complex selection history and *vinifera* back crossings, referred to as Neo-American hybrids, have subsequently been developed.[52] Both French-American and Neo-American hybrids can produce wines that exhibit organoleptic qualities atypical of *vinifera* wines and associated with wild *Vitis*. These generally undesirable traits can include excessive sourness due to high malic acid,[53] excessive herbaceousness due to

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[a] Reprinted with permission from: Springer, L. F.; Sacks, G. L., Protein-precipitable tannin in wines from *Vitis vinifera* and interspecific hybrid grapes (*Vitis* ssp.): Differences in concentration, extractability, and cell wall binding. *J. Agric. Food Chem.* **2014**, *62*, 7515-7523. Copyright 2014 American Chemical Society

odorants like methoxypyrazines,[54] and, in crosses with *V. labrusca* parentage, 'foxy'-smelling aromas due to odorants like methyl anthranilate and *o*-aminoacetephonone.[55, 56]

Another undesirable characteristic associated with red wines produced from hybrid grapes is low astringency, resulting in poor mouthfeel.[34, 57] The major compound class responsible for astringency in red wines are the CTs (proanthocyanidins).[16] CTs are polymers of flavan-3-ol subunits, mainly catechin, epicatechin, epicatechin 3-*O*-gallate, and epigallocatechin, linked by C4-C8 or C4-C6 interflavan bonds. The mean degree of polymerization (mDP), subunit composition, and extractability during winemaking of CTs is tissue specific and, in most cases, cultivar dependent.[58, 59] CTs consisting of polymers of three or more subunits are able to bind to and precipitate salivary proteins or cross link those on the oral mucosa surface, leading to an increase in friction in the oral cavity and the sensation of 'astringency', while monomers and dimers are reported to have low astringency and greater bitterness.[21, 60, 61] In addition to affecting wine mouthfeel, CTs can also stabilize wine color through polymeric pigment formation via reaction with anthocyanins and other wine components.[62] Because red wines with low CT concentrations are correlated with lower bottle prices and consumer liking,[24] selecting for grapes capable of producing higher CT wines could be a target for grape breeders that would confer value to improved cultivars.

Recent studies, though limited in scope, have indicated that CT concentrations are substantially lower in red wines produced from hybrid grapes than from *vinifera* and that these differences arise from grape composition rather than production practices. For example, using the Adams-Harbertson protein precipitation assay, CT concentrations of <100 mg/L catechin equivalents (CE) were observed in wines produced from the hybrid grapes Corot noir and Marèchal Foch, as compared to an average of 500 mg/L in *vinifera* wines.[48, 49, 63] Furthermore,

vinification techniques aimed at increasing hybrid wine CT, including extended maceration, pectinase enzyme addition, and exogenous CT addition, were shown to be ineffective.[34] However, concentrations of CT in the skin and seeds of the hybrid grapes were comparable to literature reports of CT in red *vinifera* grapes.[48, 49] While interpretation of these results are complicated by the fact that the studies were performed in different regions and with different winemaking protocols, they suggest that variation in wine CT may result from differences in grape CT extractability rather than grape CT concentration. The impact of CT extractability on final wine CT concentrations has been recognized in studies on *vinifera*. In contrast to anthocyanin pigments, CTs in grapes are reported to be uncorrelated with CTs in finished wines – even under identical winemaking conditions.[64] Multiple authors have reported that a significant portion of grape CT remains bound to insoluble cell wall materials after fermentation, and that the ability of both berry skin and flesh cell walls to bind CT could account for the incomplete extraction of tannin into wine.[40, 64] Potentially, greater tannin-cell wall interactions and thus poorer tannin extractability could also explain low CT concentrations in red hybrid wines.

In this work, we first confirmed that CT concentrations were significantly lower in wines produced from hybrid grapes in comparison to *vinifera*, as had been observed in limited previous reports. We then evaluated two separate hypotheses to explain these differences, either that the grape species differed in CT concentration or that they differed in CT extractability. The latter was investigated by quantifying CT-binding by grape cell wall material, and appeared to be the more important explanation. Finally, we hypothesized that differences in CT binding among cell wall fractions could be related to differences in the major CT-binding macromolecular classes, protein and pectin.

MATERIALS AND METHODS

Chemical Reagents

Ethanol (reagent grade), (+)- catechin hydrate, albumin from bovine serum, iron (III) chloride, triethanolamine (TEA) sodium dodecyl sulfate (SDS), ViscozymeL®, sodium tetraborate, 3-phenylphenol, sulfamic acid, and D-(+)- galacturonic acid monohydrate were obtained from Sigma Aldrich (St. Louis, MO). Acetic acid, sodium hydroxide, hydrochloric acid, potassium metabisulfite, acetone, L-(+)-tartaric acid, Clinitest tablets (Bayer, Pittsburgh, PA), sodium acetate anhydrous, and sulfuric acid were sourced from Fisher Scientific (Waltham, MA). A grape tannin extract, BioTan, was provided by Laffort (Petaluma, CA). Diammonium hydrogen phosphate (DAP) was purchased from Presque Isle Wine Cellars (North East, PA). GoFerm Protect, Fermaid K, and Lalvin ICV-GRE yeast were obtained from Lallemand (Santa Rosa, CA).

Survey of CTs in V.Vinifera *vs. Hybrid (*Vitis spp.*) Wines*

Ten commercial *vinifera* varietal wines and 10 interspecific hybrid wines from the Finger Lakes (NY) AVA were purchased from a local store (Geneva, NY). Wines covered the vintages 2010-2012. Additionally, two *V. vinifera* varietals and two interspecific hybrid wines from these vintages were provided by the Cornell Vinification and Brewing facility (Geneva, NY). Protein precipitable CT was measured using the Adams-Harbertson assay, as described elsewhere.[30]

Grape Sample Collection

Six *vinifera* cultivars (Pinot noir, Merlot, Lemberger, Sangiovese, Cabernet Sauvignon, and Cabernet franc) and six *Vitis* spp. hybrids (French-American hybrids: Baco noir, Leon Millot, Maréchal Foch, and DeChaunac; Neo-American hybrids: Corot noir and Noiret) were hand harvested across two vineyard locations within the Finger Lakes AVA in NY State at

commercial harvest time points in 2012. Three kg of each cultivar was collected, from which 50 berries were randomly selected and pressed for juice analysis. Titratable acidity was measured using a Titrino Plus 848 doser and 869 autosampler (Metrohm USA, Riverview, FL), soluble solids were determined with an Atago digital pocket refractometer (Japan), and pH was analyzed using an Accumet Excel XL25 pH Meter (Thermo Fisher Scientific, Waltham, MA). Harvested fruit not used for winemaking was kept frozen at -20 °C for further analyses.

Winemaking

From each harvested sample, fresh berries were manually destemmed, and 750 g was crushed in 1 L fermenters in duplicate. Musts were treated with 100 mg/L sulfur dioxide, added as potassium metabisulfite, before inoculating with yeast (0.2 g/L, rehydrated with GoFerm according to manufacturer's instructions). Fermaid K and DAP were added at a rate of 0.25 g/kg and at 0.3 g/kg after 48 h. All samples were fermented at 18 ˚C on the skins with daily punch downs. After 7 days, residual sugar was determined to be below 0.5 g/L in all fermentations by Clinitest and wines were pressed manually using cheesecloth. Wines were supplemented with 60 mg/L sulfur dioxide, bottled in 187 mL glass bottles under nitrogen, and crown capped (Waterloo Container, Waterloo, NY). Bottled wine was stored in the dark at 2 °C until analyzed.

Condensed Tannin

Measurements on Experimental Wine and Grape Samples

CT in wine was measured by the Adams-Harbertson protein precipitation assay as described elsewhere.[30] Samples that fell below the limit of quantification (100 mg/L, or an absorbance change of <0.3 AU [65]) were concentrated in an Eppendorf vacufuge (Hamburg, Germany) until the volume was reduced by half, and the protein precipitation assay re-run. To determine CT in fruit, a frozen 50 berry sub-sample (in duplicate) was dissected on ice into skin, flesh, and seed

fractions using forceps and a scalpel. Freshly dissected sample weights of each tissue were recorded. An extract was prepared from the skins by agitation in 100 mL of 70% (v/v) acetone overnight, and solids were separated by vacuum filtration and saved for cell wall material preparation. Acetone was removed from the extract under reduced pressure at 30 °C and volumes were adjusted to 1 mL with filtered water (Millipore Corporation, Billerica, MA) before measuring protein precipitable CT. Seeds were extracted with 25 mL of 70% (v/v) acetone overnight with agitation. Solids were filtered as for skins, acetone was removed, and the final volumes were adjusted to 1 mL with MilliQ filtered water before measuring protein precipitable CT. CT extractability was calculated as the ratio of protein precipitable CT quantities (mg) in a volume of wine (L) to protein precipitable CT quantities in a corresponding weight of berries (mg/g, skin plus seed):

Equation 2.1

$$\text{CT Extractability} = \left(\frac{Wine\ CT\frac{mg}{L}CE \times Wine\ Yield\ (L)}{\left(Seed{+}Skin\ CT\frac{mg}{g}CE\right) \times Initial\ Must\ Weight(g)} \right) \times 100\%$$

Preparation of Alcohol Insoluble Cell Wall Material

Cell wall material was prepared as alcohol insoluble solids in a manner similar to de Vries and coworkers.[66] For each 50 berry sub-sample replicate, acetone extracted skins (from above) were extracted in 200 mL of hot 95% (v/v) ethanol for 10 min and vacuum filtered. The procedure was repeated twice for each replicate. The alcohol insoluble cell wall material was then rinsed with acetone and allowed to dry overnight in a 40 °C oven, weighed, ground to a fine powder using a Genogrinder 2000 (Spex SamplePrep, Metuchen, NJ), and stored at -20 °C until use. This procedure was repeated for the grape flesh fractions, isolated as described in the previous section.

Evaluation of Condensed Tannin Binding by Cell Wall Material

CT binding experiments were performed in model wine, consisting of 1.5 g/L BioTan, 12% (v/v) ethanol, 5 g/L tartaric acid, and MilliQ filtered water with the pH adjusted to 3.3 using NaOH. Ten mg of each skin or flesh cell wall fraction was weighed into a 1.5 mL microcentrifuge tube in duplicate. To each tube, 1 mL of the BioTan model wine solution was added. Controls containing only the BioTan model wine and no cell wall material were prepared in triplicate. Microcentrifuge tubes were sealed with Parafilm® and incubated overnight at room temperature (23 °C) on a shaker in the dark to allow for CT– cell wall binding, after which insoluble material was pelleted by centrifugation at 10,000g for 5 min. The protein precipitable CT was then measured for the supernatant from each tube. For each tube, the amount of CT bound to cell wall material was calculated as the difference between the protein precipitable CTs measured in the controls and the tube's supernatant. For this paper, a distribution coefficient, K, was used to quantify the degree of CT binding where a lower K value indicates stronger binding. K was calculated by dividing the concentration of CT remaining in the model wine solution after incubation with cell wall materials by the apparent concentration of CT bound to cell wall material, Equation 2.2:

$$K = \frac{[CT\ in\ Supernatant]}{[CT\ in\ Control - CT\ in\ Supernatant]}$$

Protein and Pectin in Cell Wall Materials

Percent nitrogen was determined for all cell wall samples using a Thermo Delta V isotope ratio mass spectrometer interfaced to a NC2500 element analyzer at the Cornell Isotope Laboratory (Ithaca, NY). Crude protein was calculated as mg/g nitrogen x 5.27.[67] Pectin was measured as the uronic acid content following enzymatic digestion. Enzymatic digestion was selected because it yields superior galacturonic acid recovery with less degradation in

comparison to traditional hot acid hydrolysis.[68] Briefly, 5 mg of cell wall material was incubated in 950 µL of 25 mM acetate buffer, pH 4.8, in a screw capped 1.5 mL microcentrifuge tube. Ten µL of Viscozyme was added to each tube. Samples were mixed by vortexing and incubated at 30 °C for 18 h on a shaker. After incubation, samples were centrifuged at 13,000 rpm for 5 min and supernatant containing uronic acids was collected for analysis. Uronic acids were quantified using a spectrophotometric method with D-galacturonic acid as standard, as previously described.[69]

Statistical Analysis

For the Finger Lakes wine survey, samples that fell below the limit of quantification (100 mg/L CE, or an absorbance change < 0.3 AU, [65]) were assigned a value of half the quantification limit (50 mg/L CE) for statistical testing.[70, 71] A similar approach was taken for experimental wines with unquantifiable CT, except that the quantification limit was 50 mg/L CE because of the concentration step, and a value of 25 mg/L CE was assigned to these wines for statistical purposes.[70, 71] JMP Pro 10 (by SAS, Cary, NC) was used to perform all statistical testing and modeling for this study, with a type 1 error rate set to 0.05. Student's t-test was performed to compare *vinifera* and hybrid wine CT in the commercial wine survey. One-way ANOVA was performed on grape tissue weights, cell wall yields, skin CT, seed CT, total CT, CT extractability, CT binding cell wall experiments, and for measurements of protein and pectin. Where significant differences were observed following ANOVA, Tukey's range test was performed to determine if differences existed between individual groups. To investigate the effects of cultivar, harvest location, and grape classification on wine CT, a multivariate fixed effects modeling approach was used to identify significant variables. Graphics were generated using GraphPad Prism version 5.0c (GraphPad Software, San Diego, CA).

RESULTS AND DISCUSSION

Recent reports have indicated that hybrid-based wines have low CT concentrations as compared to literature reports for *vinifera* varietal wines, but these studies have been limited in scope.[34, 48, 49] We performed a survey consisting of hybrid- and *vinifera*-based wines, mostly commercial, using the Adams-Harbertson protein precipitation assay to quantify CTs (Supplementary Figure S2.1, Appendices). The Adams-Harbertson assay was selected over other methods, e.g those that measure total proanthocyanidins, because it is reported to best correlate with perceived astringency and thus wine flavor,[20] although one challenge was the relatively high limit of quantification (LOQ). The average protein precipitable CT was significantly higher in *vinifera*- than hybrid-based wines (255 vs 61 mg/L CE, $p < 0.05$). Furthermore, ten out of 12 of these wines fell below the limit of quantification (100 mg/L CE [65]), and only one hybrid-based wine contained >200 mg/L CE of tannin. By comparison, only three of 12 *vinifera*-based wines in our survey had <200 mg/L CE of tannin, and a larger survey of red *vinifera*-based wines observed only about 8% had <200 mg/L CE of CT.[63]

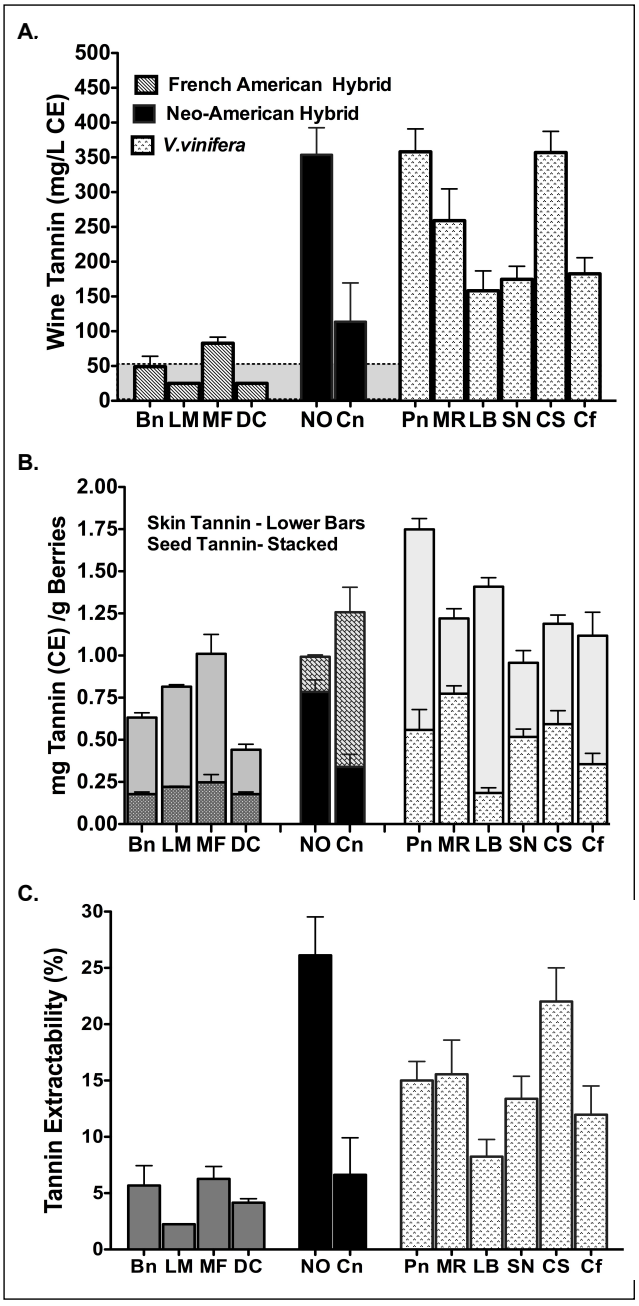
The CT concentrations of *vinifera* wines in our commercial wine survey were approximately 50% of that measured by the same assay in red wines from CA, WA, OR and Australia, 544 mg/L CE.[63] While the distribution of varietal wines included in the two surveys was different, lower concentrations were typically observed for the same varietal wine. For example, the average protein precipitable tannin in Cabernet Sauvignon wines from Washington State averaged 660 mg/L CE, while the Cabernet Sauvignon wines included for this survey from the Finger Lakes averaged 266 mg/L CE. The Finger Lakes region of NY is a cool climate region having less growing degree-days, heat summation, sunlight, and more precipitation than many of the aforementioned warmer regions.[72] Several of these factors are reported to decrease grape or

wine CT concentrations. For example, increases in heat summation between fruit set and veraison has been associated with increased CT in Pinot noir berries and their respective wines [58], and greater water availability has been linked to decreases in flavonoid biosynthesis.[73]

Wine Condensed Tannin in Experimental Wines Produced from Different Cultivars

Because winemaking protocols can have a profound effect on wine CTs, an alternative explanation for observed differences in CTs between *vinifera* and hybrid-based wines could be that commercial wineries utilize different practices for these grapes. Additionally, US labeling laws allow the addition of 25% of varieties other than those listed on the label. To control these variables, we collected fruit from 12 cultivars (four French-American hybrids, two Neo-American hybrids, and six *vinifera* cultivars), with each cultivar collected from two different sites for a total of 24 grape lots. Basic fruit chemistry is shown in Supplementary Table S2.2 (Appendices). Fruit was then vinified using a standardized small-scale winemaking protocol and CT quantified in the finished wines using the Adams-Harbertson assay (Figure 2.1A).

FIGURE 2.1 Condensed Tannin in Fruit, Wines, and the Percent Extracted During Fermentation



A. Protein precipitable wine CT by variety (n=4 per variety, bars indicate standard error of the mean) Bn= Baco noir, LM= Leon Millot, MF= Marechal Foch, DC= DeChaunac, NO= Noiret, Cn= Corot noir, Pn= Pinot noir, MR= Merlot, LB= Lemberger, SN= Sangiovese, CS= Cabernet Sauvignon, Cf= Cabernet franc
B. Protein precipitable CT per gram of berries by cultivar
C. CT extractability by variety

A multivariable fixed effects model fitting wine CT with variables "classification" (French American hybrid, Neo-American hybrid, or *vinifera*), "cultivar" nested within classification, and "location" revealed that location did not have a significant influence on wine CT ($p=0.51$). Cultivar was a significant factor ($p<0.05$) in the model, similar to other findings.[74] Additionally, belonging to a classification group of "French American hybrid", "Neo American hybrid" or "*vinifera*" was a significant determinant of wine CT ($p<0.05$). Wines produced from classic French-American hybrid grapes had significantly lower CT concentrations than *vinifera*-based wines based on mean values, 45 mg/L CE vs. 248 mg/L CE respectively (5.5 fold lower, $p<0.05$). Even though winemaking practices were kept constant, the range in wine CT from these two classes reached a maximum variation over 17.4 fold. Notably, CT in both *vinifera* and hybrid-based wines were comparable to data in Figure 2.1, indicating that our experimental wines were comparable to commercial wines.

The Neo-American hybrids had higher CT concentrations than the classic French-American hybrids. Noiret had the highest amount of CT out of all hybrid-based wines, averaging 354 mg/L CE. The other Neo-American hybrid in this study, Corot noir, was variable, with Corot noir wine produced from fruit from one site having 203 mg/L CE wine CT while Corot noir from the second site had CT below LOQ for the protein precipitation assay.

Although the Neo-American hybrids investigated have a similar percentage of *vinifera* in their background as the French-American hybrids (~50%), the pedigrees of most Neo-American hybrids involve several back-crosses, including Corot noir, Noiret, and Vincent (Wine 22 from Supplementary Figure S2.1).[52] In contrast, many classic French-American hybrids result from a direct cross (F_1) of *vinifera* with a wild *Vitis* species (or crosses of wild *Vitis*). A potential explanation for low wine CT in the classic French-American grapes is that traits that lead to

higher wine CT are derived from *vinifera* and recessive, and that back-crossing is necessary for hybrid grapes to yield high wine CT.

Relation of Grape Condensed Tannin and Condensed Tannin Extractability to Wine Condensed Tannin in Experimental Wines

To evaluate if differences in wine CT concentrations arose from differences in grape CT, the CT in skins or seeds was quantified. These data are reported on a per berry weight basis in Figure 2.1B. Skin and seed CT among grape classes were evaluated by a one-way ANOVA and were significantly different ($p<0.05$). Total CT (skin + seed) concentrations were significantly higher in *vinifera* grapes (mean = 1.27 mg CE/g berry) as compared to French-American hybrid grapes (0.71 mg CE/g berry). CT concentrations in vinifera grapes were similar to previous reports using the same protein precipitation method. Harbertson and colleagues reported values from Cabernet Sauvignon, Pinot noir, and Syrah ranging from 0.99-1.44 mg CE/g berry.[30] Although the 1.8-fold higher total CT concentrations in *vinifera* grapes may serve as a partial explanation for differences in wine CT, it cannot fully account for the 5.5-fold higher CT concentrations (and 17 fold maximum difference vs 6.1 fold maximum difference in berry CT) between *vinifera*- and French-American hybrid-based wines. This indicates that the cultivar classifications must also vary in CT extractability.

CT extractability was calculated as described in Equation 1 and data are shown in Figure 2.1C. Extractability from French-American hybrids was lower than that of the other two classes (1-way ANOVA, $p<0.05$) and ranged from 2.2-5.7% in French-American hybrids, 6.6-26.1% in Neo-American hybrids, and 8-22% in *vinifera*. The low CT extractability observed from French-American hybrids is comparable to previous reports from our group,[48, 49] and the extractability observed for *vinifera* is on the lower end reported by others (4.9-61%).[30]

We observed that skin CT was better correlated with final wine CT across cultivars (r^2 = 0.73, $p<0.05$), as compared to total CT (r^2=0.44, $p<0.05$) and seed CT (ns) (Table 2.1).

TABLE 2.1 Correlations Between Fruit Condensed Tannin, Wine Condensed Tannin and Condensed Tannin Extractability

Predictor	Wine CT			CT Extractability		
	p value	r^2	RMSE	p value	r^2	RMSE
Total CT	0.0179	0.44	99.7	0.21	0.15	7.08
Skin CT	0.0004	0.73	69.1	0.0001	0.79	3.52
Seed CT	0.75	0.01	133	0.51	0.04	7.52

Thus, differences in grape skin CT may partially explain both differences in wine CT and apparent CT extractability during fermentation. The median concentration of skin CT in *vinifera* is 2.7-fold higher than in French-American hybrids (0.54 vs. 0.20 mg CE/g berry). Skin CT is extracted more quickly than seed CT, with one report stating that skin CT extraction reaches a maximum after five days, while seed CT extraction occurs more slowly and does not reach equilibrium by the time the fermentation is at dryness.[58] Assuming 29% skin CT and 6% seed CT extraction during maceration (excluding any other calculated binding effects) as previously reported by Kennedy,[75] the predicted average French American hybrid wine would be 126 mg/L CE while the average vinifera wine would be 261 mg/L CE, a 2.1-fold difference. However, *vinifera*-based wines averaged 5.5-fold higher CT concentrations than French-American hybrids (Figure 2.1A), indicating that other factors beyond skin CT likely contribute to differences in CT extractability.

Condensed Tannin Binding by Cell Wall Material

Factors that contribute to variation in CT extraction among grape sources under consistent winemaking conditions are not fully understood, but binding of CTs by cell wall material has been proposed.[40] We observed 17% more skin tissue by weight for *vinifera* vs. French American hybrids, while cell wall yields from skins by fresh weight were not significantly different (Table 2.2). A small, but significant, increase in flesh cell wall material yield was observed for French American hybrids than *vinifera* (12.3 mg/g vs 10.2 mg/g fresh weight, Table 2.2). The Neo-American hybrid grapes had significantly more skin cell wall material, but not flesh cell wall material, than both vinifera and French American hybrid fruit by fresh tissue weight (56.0 mg/g vs 46.7 mg/g and 44.9 mg/g, respectively, Table 2.2). Because differences in cell wall material quantity were small, they were thought unlikely to affect extractability.

TABLE 2.2 Skin and Flesh Cell Wall Yields and Composition by Cultivar

Cultivar	Tissue	Fresh Weight (mg/g Berries)	Cell Wall Yield (mg/g FW)	Crude Protein (mg/g CW)	Pectin (as uronic acids) (mg/g CW)	Distribution Coefficient (K)
Baco noir	skin	212 ± 5.5 c	38.7 ± 0.91 e	113 ± 2.5 a	178 ± 3.6 a b c	6.13 ± 0.052 d
	flesh	705 ± 5 w x y z	11.7 ± 0.59 x y z	98.7 ± 0.92 z	71.3 ± 2.1 x y z	8.48 ± 0.73 y
Leon Millot	skin	230 ± 17 a b c	45.8 ± 2.8 b c d e	106 ± 3.3 a	192 ± 11 a b	8.40 ± 0.57 c d
	flesh	667 ± 19 x y z	9.25 ± 0.96 y z	102 ± 5.0 y z	91 ± 3 x y	7.78 ± 0.86 y
Marechal Foch	skin	211 ± 17 c	48.3 ± 1.4 b c	116 ± 2.5 a	173 ± 9.4 a b c	8.30 ± 0.088 c d
	flesh	672 ± 23 w x y z	15.1 ± 1.0 x	119 ± 4.2 x y	71.5 ± 2.2 x y z	8.75 ± 0.60 x y
DeChaunac	skin	199 ±11 c	46.7 ± 0.97 b c d e	103 ± 3.1 a	227 ± 8.0 a	9.74 ± 0.71 b c d
	flesh	737 ± 16 w	13.0 ± 0.48 x y	90 ± 2.6 z	68.3 ± 4.5 x y z	10.1 ± 0.52 w x y
Average	**Skin**	**213 ± 6.7 B**	**44.9 ± 2.1 B**	**110 ± 1.9 A**	**193 ± 6.6 A**	**8.14 ± 0.40 B**
	Flesh	**695 ± 11 X**	**12.3 ± 0.66 X**	**102 ± 6 X Y**	**75.5 ±2.7 Y**	**8.78±0.38 Y**
Noiret	skin	279 ± 7.8 a	47.5 ± 0.85 b c d	103 ± 4.6 a	159 ± 20 b c	15.6 ± 1.1 b c d
	flesh	665 ± 7.8 x y z	10.7 ± 0.97 x y z	84.7 ± 4.1 z	99 ± 5 x	11.8 ± 0.99 w x y
Corot noir	skin	200 ± 8.3 c	64.4 ± 1.8 a	111 ± 4.8 a	172 ± 11 a b c	10.8 ± 0.70 b c d
	flesh	731 ± 9.8 w x	11.5 ± 1.7 x y z	138 ± 6.7 x	84.5 ± 2.4 x y z	2.99 ± 1.1 z
Average	**Skin**	**239 ± 16 A B**	**56.0 ± 8.5 A**	**107 ± 3.4 A B**	**166 ± 11 A B**	**13.2 ± 1.1 B**
	Flesh	**698 ± 14 X**	**11.2 ± 0.92 X Y**	**112 ± 27 X**	**91.8 ± 3.8 X**	**7.39±1.8 Y**
Pinot noir	skin	270 ± 7.8 a b	49.7 ± 1.3 b	102 ± 6.2 a	121 ± 5.0 c	36.5 ± 9.1 a
	flesh	668 ± 8.9 x y z	8.47 ± 1.3 z	103 ± 4.5 y z	62.9 ± 3.7 y z	9.59 ± 0.86 w x y
Merlot	skin	252 ± 21 a b c	39.4 ± 2.7 d e	100 ± 3.4 a	168 ± 5.0 b c	17.9 ± 1.6 b c d
	flesh	675 ± 24 w x y z	10.1 ± 0.68 y z	84.0 ± 2.5 z	62.7 ± 1.0 y z	11.6 ± 0.82 w x y
Lemberger	skin	212 ± 4.5 c	61.2 ± 1.7 a	107 ± 5.8 a	140 ± 6.6 b c	20.5 ±0.74 b c
	flesh	733 ± 7.3 w x	10.9 ± 0.56 x y z	98.7 ± 3.6 z	67.4 ± 5.3 y z	13.4 ± 0.83 w
Sangiovese	skin	220 ± 12 b c	44.5 ± 1.7 b c d e	104 ± 0.84 a	177 ± 27 a b c	22.8 ± 2.0 a b
	flesh	726 ± 14 w x y	8.10 ± 0.36 z	87.0 ± 2.1 z	73.0 ± 4.6 x y z	12.8 ±1.3 w x
Cabernet Sauvignon	skin	274 ± 3.9 a b	41.1 ± 0.69 c d e	104 ± 3.4 a	148 ± 6.1 b c	19.5 ± 2.0 b c d
	flesh	659 ± 4.0 y z	10.7 ± 0.61 x y z	99.2 ± 4.7 z	54.6 ± 4.2 z	13.3 ± 0.94 w
Cabernet franc	skin	272 ± 4.5 a b	44.1 ± 1.5 b c d e	95.6 ± 7.0 a	171 ± 6.2 a b c	15.5 ± 0.60 b c d
	flesh	641 ± 7.4 z	13.1 ± 0.89 x y	98.3 ± 2.8 z	87.1 ± 18 x y	10.5 ±0.31w x y
Average	**Skin**	**250 ± 6.5 A**	**46.7 ± 3.2 B**	**102 ± 1.9 B**	**154 ± 6.1 B**	**22.1 ± 2.0 A**
	Flesh	**684 ± 8.5 X**	**10.2 ± 0.45 Y**	**95 ± 3 Y**	**68.0 ± 3.6 Y**	**11.9 ± 0.44 X**

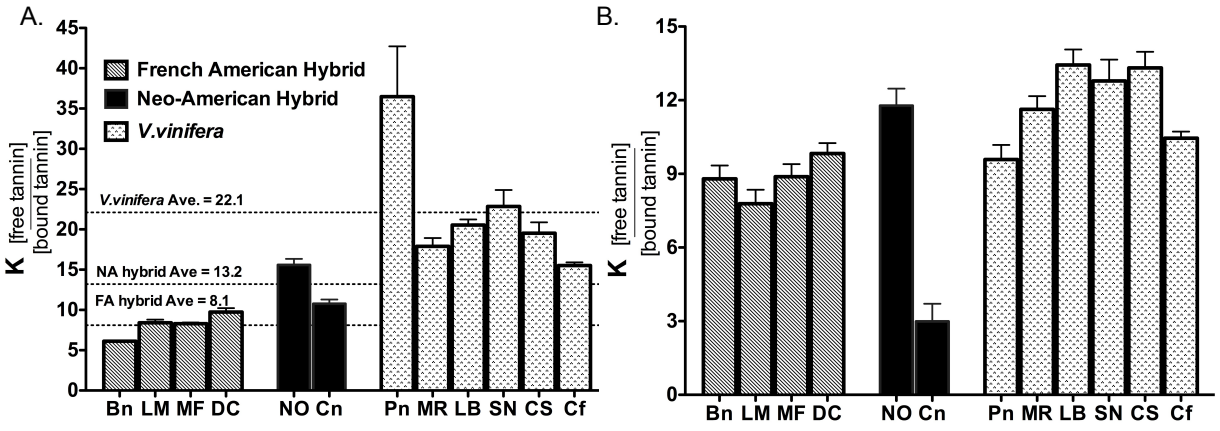
Significant differences ($p<0.05$) by Tukey-Kramer HSD among cultivars are denoted by not sharing lower case letters for skin tissue (a, b, c, d) or flesh tissues (w, x, y, z). Significant differences among classes (French-American, Neo-American, *V. vinifera*) are indicated by capital letters for skin tissue (A, B, C) or flesh tissue (X, Y, Z).

To investigate CT binding affinity rather than cell wall material concentration could contribute to differences in wine CT among the classes, alcohol insoluble residues were prepared from skins and flesh from each grape source. The protocol selected was previously employed for preparing cell wall material samples from *vinifera* grapes and results in isolation of

carbohydrates, proteins, phenolic compounds, and cell wall microstructure with lower cost and preparation time per sample than comparable methods.[76]

Equal masses (10 mg) of cell wall material were incubated with a commercial CT product (BioTan) in a wine-like medium to evaluate CT binding of each fraction. BioTan was selected for this study based on previous work that showed it had the highest concentration of iron reactive protein precipitable CT (48%) for commercial grape derived products.[33] In close agreement with Harbertson and coworkers, we calculated the iron reactive protein precipitable CT to be 47% by weight.[33] Phloroglucinolysis analysis by other authors indicates that BioTan is more similar to seed CT than skin in size (mDP = 6.93).[77]

Figure 2.2 Distribution Coefficients of Partitioned CT in Skin and Flesh Cell Walls



Calculated as ratio of free to bound CT, n=8, bars indicate standard error of the mean
A After an 18-hour incubation of CT with skin cell wall
B. After an 18-hour incubation of CT with flesh cell wall

Skin cell wall materials from French-American hybrid grapes were able to bind significantly more CT on a by weight basis than *vinifera* ($p<0.05$, Figure 2.2A). For this paper, we define the binding affinity of skin cell wall material as a distribution coefficient (K), calculated as the ratio of CT in model wine vs. the skin cell wall material during incubation experiments. K was 2.7 fold greater in *vinifera* than in French-American hybrids. The ability of Neo-American hybrid

skin materials to bind CTs was not significantly different from *vinifera* or the French-American hybrids, and the average distribution coefficient fell between the other two groups. Flesh cell wall materials from all three classes were not significantly different in their binding ability (Figure 2.2B, $p>0.05$). Although CT binding by cell wall material from Neo-American grapes did not differ significantly from French American and *vinifera* grapes, Noiret flesh demonstrated significantly less binding than both Corot noir and the French-American hybrids (Figure 2.2). We also observed that the Corot noir flesh cell wall sample with the highest binding affinity corresponded to the Corot noir wine with undetectable CT. While cell wall binding could potentially explain the greater CT extractability observed in Noiret as compared to Corot noir (Figure 2.1C), Noiret also had significantly greater skin CT (Figure 2.1C), and it is not currently possible to decouple which of these effects is more important.

As a caveat, these distribution coefficients are specific for the test conditions employed. CT-cell wall interactions do not follow simple Langmuir or Freundlich binding patterns and may involve cooperative binding.[78] Using a different CT preparation (e.g. a skin-derived CT fraction) would be expected to lead to different absolute K values, and may reveal additional differences in the CT binding behavior between cell wall fractions.[43] Given that skin CT was found to be a significant factor in determining final wine CT, further characterizing the interaction of cell wall material with skin CT would benefit our current understanding of CT extractability. Additionally, we used cell wall materials from grapes, which may not account for cell wall changes that occur during fermentation. However, our work does appear comparable to Bindon and colleagues, who recently reported on CT binding by Shiraz cell wall material.[43] In agreement with their work, *vinifera* flesh cell wall materials in our study bound significantly more CT than skin cell wall material. Although this previous work expressed binding in different

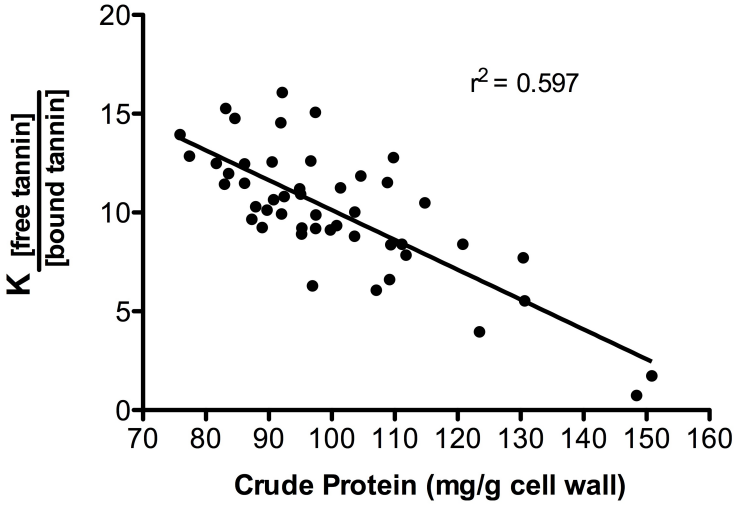
units than in our current report, it was possible to determine distribution coefficients from the data presented.[43] We calculate that the Shiraz-derived cell wall material incubated with seed CTs had K values of 17 (flesh) and 30 (skin), comparable to the values reported here for *vinifera*.

Characterization of Cell Wall Material: Protein and Pectin Content

Since the reasons for differences in CT binding among classes or cultivars could arise from differences in grape composition and/or structure, we quantified the two major classes of CT-binding macromolecules, pectin and protein.[43, 79] Pectin was quantified as uronic acids (in galacturonic acid equivalents) presuming galacturonic acid is the main constituent of grape pectin and glucuronic acid is a minor constituent of grape cell walls (Table 2.2).[80, 81] The gravimetric recovery of cell wall materials reported here is within the range of values previously reported.[67] Pectin and crude protein quantities in both skin and flesh cell wall materials are also within the range of other reports using similar methods of quantification.[67] We observed that French-American hybrid grape skin cell walls had significantly more pectin (as uronic acids). Pectin has been shown to bind CT more efficiently than other less flexible carbohydrates,[41, 67, 82] and pectin differences may contribute to observed differences in extraction from French-American hybrids. We also observed that pectin content was weakly correlated with K distribution coefficients ($p<0.05$, $r^2= 0.255$). As a caveat, the pectin molecular weight and degree of substitution was not analyzed in this study and may account for additional small differences in CT binding affinity.

An even stronger correlation was observed between K values and flesh protein ($p<0.05$, $r^2 = 0.597$, Figure 2.3).

FIGURE 2.3- Flesh Cell Wall Distribution Coefficients (K) vs Corresponding Cell Wall Crude Protein Content



As a specific example, Corot noir flesh contained the highest amount of protein by weight, and the lowest K values (strongest binding). This is in agreement with Bindon, et al, who observed that crude protein was higher in Cabernet Sauvignon cell wall fractions possessing greater CT affinity.[67] French American hybrid skin cell walls also had higher crude protein than *vinifera,* though differences were small and the correlation coefficient of K and skin protein was worse ($p<0.05$, $r^2= 0.19$). Protein concentrations in our study ranged from 8-14%, although these values may be higher due to precipitation of cytoplasmic proteins with the ethanol precipitation method of cell wall preparation.[40, 83] The interaction between structural cell wall proteins, such as the hydroxyproline rich extensin, and CT have been recently discussed, but their impact on wine CT has not been thoroughly investigated.[40] Because of the nature of this study, it is not possible to determine if flesh protein content is a direct cause of low CT extractability or merely correlated.

In agreement with previous studies using *vinifera* cultivars, flesh cell walls bound more CT than skin cell walls in this classification, but had less pectin and protein by weight in comparison in this study.[43] This could be due to the presumably more flexible nature of flesh cell wall material facilitating stronger CT interactions, but additional information is needed to address this. Bindon and colleagues reported that cell wall flexibility was an important aspect for CT adsorption, increased adsorption with decreasing acid-insoluble fiber and insoluble bound CT was reported for Cabernet Sauvignon,[67] but these two parameters were not quantified in this study. Thus, although pectin and protein content may contribute to CT extraction differences among cultivars, more detailed compositional analysis may be necessary to understand differences in CT binding between tissues.

This publication did not evaluate all possible factors that could affect CT extractability. For example, the French-American hybrids are reported to have high anthocyanin concentrations as compared to vinifera.[84, 85] While anthocyanins can bind cell walls,[86] a competitive binding effect seems unlikely because low anthocyanin concentrations are reported to decrease CT extraction.[45, 87] Differences in CT composition, particularly CT size (mDP) and, to a lesser extent, degree of galloylation, could also decrease extraction by increasing binding efficiency to cell wall material.[43] Differences in CT composition between hybrids and *vinifera* was not investigated in this work.

Potential Consequences to Winemaking with Hybrid Grapes

Our data indicate that lower CT concentrations (5.5-fold, up to a maximum 17.4 fold) are present in hybrid-based wines as compared to *vinifera*-based wines in both commercial wines and in wines produced under standardized winemaking conditions. While these differences can be partially explained by differences in berry CT (particularly skin CT), differences in CT

binding by cell wall material appear to explain an equal or greater amount of the variation. The demonstrated importance of skin CT to final wine CT in this report in conjunction with the timing of skin CT extraction during winemaking reported elsewhere, emphasize the critical impacts of cell wall interactions on final wine CT. The high CT-binding of hybrid grapes may explain the lack of success observed with winemaking approaches designed to increase wine CT, especially in the early stages of winemaking. For example, recent work on hybrid grapes observed that pre-fermentation CT additions to must and extended maceration resulted in no significant increase in wine CT.[34] Additionally, the mDP of hybrid wine CT in this previous work was low as compared to *vinifera*-based wines (mDP < 4) and decreased throughout the course of fermentation. These low molecular weight CTs are anticipated to contribute more bitterness than astringency. Other work on *vinifera* has demonstrated selective removal of high mDP CTs by cell wall materials, resulting in lower mDP for CTs remaining in solution.[43] Since skin CT has both higher mDP and is extracted more rapidly than seed CT during fermentation, there will be a preferential loss of skin CT due to cell wall binding.[43] Thus, the higher binding affinity of hybrid grape cell wall material may not only account for low CT, but also for a reduction in mDP. Furthermore, removing or degrading the CT-binding compounds may prove more effective for increasing final CT concentrations in these wines (hybrid or *vinifera*) than commercial CT addition.

Finally, identification of grape components that limit CT extractability may make for an interesting target to facilitate breeding of high quality winegrapes. Interspecific hybrids are of interest to the grape and wine industries due to their better economic and environmental sustainability. Modern genetic tools, such as marker-assisted selection, have been used to facilitate grape breeding, especially for viticulturally important traits like disease resistance.

While a few genetic markers for wine quality related attributes have been identified, such as for herbaceous-smelling methoxypyrazines,[88] wine CT concentrations pose a particular challenge because they are poorly correlated with grape concentrations and thus are not currently well suited for high throughput phenotyping in grape mapping populations. Future work to identify compounds that limit CT extraction from grapes could facilitate these studies.

CHAPTER 3

PATHOGENESIS-RELATED PROTEINS LIMIT THE RETENTION OF CONDENSED TANNIN ADDITIONS TO RED WINES[b]

INTRODUCTION

Polymers of flavan-3-ols, CTs, impart critical organoleptic qualities to red wines, modulating the tactile sensation of red wine astringency through their interaction with salivary proteins and the oral mucosa.[18] Astringency intensity is positively correlated to CT quantity and polymer size, although other compounds or compositional differences may contribute to astringency perception or its sub-qualities.[18, 19, 23] CTs also promote color stability through their reactions with anthocyanins (colored pigments in wine) and reactive oxygen species or oxidation products, e.g. aldehydes.[11] Perhaps as a consequence of these phenomena, higher CT concentrations are often correlated with higher perceived overall red wine quality.[24, 89]

In many winemaking countries, such as the US and Australia,[32] winemakers may legally use exogenous CTs before, during, or after fermentation as an additive to improve mouthfeel, color stability, and other sensory properties. However, a number of published studies have shown surprisingly low CT retention in finished wines following exogenous CT addition, resulting in minimal or negative effects on sensory qualities.[33-35, 37] In part, this can arise from the low purity of many CT products; one study of eight commercial CTs reported that they contained only 12-48% w/w CT based on their ability to precipitate protein.[33] However, even when investigators account for initial CT purity, poor recoveries are often observed. Harbertson, et al added 60-180 mg/L CE of a commercial mix of hydrolysable and CT post-fermentation to a Merlot wine and

[b] Reprinted with permission from: Springer, L. F.; Sherwood, R. W.; Sacks, G. L., Pathogenesis-related proteins limit the retention of condensed tannin additions to red wines. *J. Agric. Food Chem.* **2016**, *64*, 1309-1317. Copyright 2016 American Chemical Society

reported no significant increase in CT afterwards.[33] Additions well above the manufacturers recommended dosage (300 mg/L) resulted in a 79% recovery (i.e. the final CT concentration increased by 79% of the amount expected based on the addition). Addition of a second commercial CT product by the same group resulted in a 64% recovery (200 mg/L addition) and a 53% recovery (800 mg/L addition). The group also observed that CT additions at recommended rates had little to no sensory impact, while higher CT additions (800 mg/L) resulted in undesirable increases in brown color, earthy aromas, and bitterness.[33] Similarly, Bautista-Ortin et. al examined the effects of a 400 mg/L addition of commercial CT to Monastrell grapes after crush, and reported no significant difference in polymeric CT from the control (no addition) at bottling.[37] Later work by the same group using a different Monastrell vintage indicated CT retention of <50% using a similar protocol.[38] Other investigators reported that adding a commercial seed CT preparation at a rate of 200mg/L to Australian Shiraz wines, either pre-or post-fermentation, resulted in no significant difference between control and treated wines at 15 out of 18 sampling time points taken over a nearly 2 year storage period.[35] While not expressly calculated, the data suggest that CT retention was <25% of the addition.[35] Similarly, adding seed-derived CT (0.5-2.0 g/L) to a low polyphenol port wine indicated a retention of 2-5% after 8 months bottle storage, although the purity of added extract was not reported in the paper.[39] CT is known to participate in reactions with anthocyanins to stabilize and enhance color,[90] but studies examining wine pigments following CT addition are not consistent.[35] For example, Parker et al. found no significant effect on color was achieved with a 200 mg/L post fermentation addition of a commercial CT preparation to Shiraz,[35] while Bautista-Ortin et al. noted improved color density and red percentages in Monastrell wines treated with 400 mg/L CT at crush.[38]

Based on a limited number of studies, the problem of poor CT recovery appears to be particularly severe in interspecific hybrid varieties (crosses between *V. vinifera* and other *Vitis* spp.). CT additions to hybrid wines should be useful, since previous work has shown that both commercial and experimental red wines produced from interspecific hybrid varieties contain negligible CT (<50 mg/L CE) as compared to *V. vinifera* based wines from the same cool climate wine region (avg. 248 mg/L).[49, 91] However, the retention of both pre- and post-fermentation CT additions to hybrid musts or wines is poor. An addition of 200 mg/L seed CT (unknown purity) post fermentation to Cynthiana wines (*V. aestivalis*) resulted in no significant increase in total phenolics, although CT was not specifically measured.[36] In another study, Manns, et al reported <20% recovery of CT following a 400 mg/L addition of seed CT at crush.[34] In the latter study, since the additions occurred pre-fermentation, the low recovery could potentially be explained by binding of CT to grape cell wall material.[40, 43, 64] In support of this, we have recently observed that cell wall material from interspecific hybrids will bind more CT than cell wall material from *V. vinifera.*[91] Furthermore, CT binding affinity by cell walls was correlated with nitrogen concentration, suggesting that grape proteins in cell walls could be responsible for the binding and low extractability of CT during fermentation.[91] However, this would not explain why studies that perform post-fermentation CT additions report incomplete and highly variable CT recovery.[33, 35, 36]

The aim of this work was to identify components that limit retention of exogenous CT additions, particularly in wines produced from interspecific hybrid cultivars and native *Vitis* species.

MATERIALS AND METHODS

Chemical Reagents

Acetone, acetonitrile (ACN), albumin from bovine serum, ammonium bicarbonate (ambic), ammonium sulfate, apomyoglobin (from horse skeletal muscle, sequencing grade), (+)- catechin hydrate, ethanol (reagent grade), iron (III) chloride, methanol, sodium dodecyl sulfate (SDS), triethanolamine (TEA), trifluoroacetic acid (TFA), Tris-HCl and urea were obtained from Sigma Aldrich (St. Louis, MO) at the highest available purity unless otherwise specified. Acetic acid, Clinitest® tablets (Bayer, Pittsburgh, PA), dithiothreitol, glycerol, hydrochloric acid, iodoacetamide (IAM), potassium metabisulfite, L-(+)-tartaric acid, sodium hydroxide, and were sourced from Fisher Scientific (Waltham, MA). Sequencing grade modified trypsin was obtained from Promega Corporation (Madison, WI). Coomassie G-250 blue dye and β-mercaptoethanol were supplied from Bio-Rad Laboratories Inc. (Hercules, CA).

Preliminary Observations–Pellets Formed Following Purified Seed CT Addition to Wine. Protein Discovery and Identification

A seed CT extract was produced by pooling 70% acetone seed extracts generated from previously published experiments.[91] Grape varieties in the extract included equal parts by volume Baco noir, Leon Millot, DeChaunac, Maréchal Foch, Corot noir, Noiret, Cabernet franc, Cabernet Sauvignon, Merlot, Pinot noir and Lemberger. The acetone solvent was removed under a N_2 stream at 30 °C. The resultant aqueous seed CT extract was purified using solid phase extraction on HLB cartridges (Waters Corporation, Milford, MA) and analyzed by a previously published HPLC phloroglucinolysis procedure to determine mean degree of polymerization (mDP).[34] The resultant purified seed CT extract was dried under a N_2 stream at 30 °C and reconstituted in model wine (12% ethanol, 5g/L tartaric acid, pH 3.3) for wine additions. Baco

noir, Maréchal Foch, Corot noir, DeChaunac, Cabernet Franc, Merlot, Cabernet Sauvignon, and Pinot noir wines were produced from a 2012 harvest as previously published.[91]

To produce the precipitate in the above listed 2012 wines, a 2 mL sample of each of the eight wines was centrifuged at 13,000g for 5 min, and a 1 mL aliquot of supernatant combined with 50 μL of the prepared model wine-seed CT extract at 20° C. The same pooled seed extract was used for all additions. Samples were vortexed for 5 s, allowed to sit for 30 min, then centrifuged at 13,000g for 5 min. The supernatant was removed, and the resultant pellet in hybrid wine samples were redissolved by vortexing in 50 μL of TEA-urea buffer (pH 8). Proteins were visualized by a standard SDS-PAGE protocol described in the Supplementary Information (Appendices). Pellets from this experiment were not subjected to CT analysis. No pellets were observed in Cabernet Franc, Merlot, Cabernet Sauvignon, and Pinot noir samples.

After SDS-PAGE and Coomassie staining, three visible gel bands were excised by scalpel and submitted to the Proteomics and Mass Spectrometry Facility at Cornell University (Ithaca, NY) for in-gel digestion and protein identification (see Supporting Information).

Compositional Analysis of Pellets Derived from Maréchal Foch Wines

For compositional analysis of pellets, Maréchal Foch wine was clarified by centrifugation for 5 min. at 13,000g. Seed CT extract in model wine (0, 10, 25, 50, 100, or 200 μL) was added to 1 mL of Maréchal Foch wine (n=6 per addition rate) and allowed to incubate overnight. All replicates were centrifuged at 13,000g for 5 min and wine was carefully decanted off pelleted materials. No pellets were observed in control wines (no CT added). CTs were quantitated via resuspension and reaction with ferric chloride, as described above, for three of the experimental replicates for each addition rate. The additional three experimental replicates were dried overnight in a Vacufuge™ (Eppendorf, Hamburg, Germany) weighed, and pooled to obtain

sufficient materials (>0.4mg) for isotope analysis. Percent nitrogen was determined for pooled pellet samples using a Carlo Erba NC2500 element analyzer (Thermo Fisher Scientific, Waltham, MA interfaced to a Delta V™ Isotope Ratio Mass Spectrometer (Thermo Scientific) at the Cornell Isotope Laboratory (Ithaca, NY). Crude protein content was calculated as µg/mg nitrogen × 6.25 × pellet weight in mg.

Grape Sample Collection and Winemaking for Condensed Tannin Retention Experiments

Cultivars of *V. aestivalis, V. cinerea,* and *V. riparia* were harvested between ~19-22° Brix from the Cold-Hardy Grape Collection, Plant Genetics Resources Unit, USDA-ARS (Geneva, NY) during the 2013 growing season. Interspecific hybrids (*Vitis* spp.) Baco noir, Chancellor, DeChanuac, and Maréchal Foch, along with *V. vinifera* cultivars Cabernet Sauvignon, Cabernet Franc, Merlot and Pinot noir, were harvested between ~17-23° Brix (see Supplementary Table S3.1, Appendices) from commercial vineyards in NY's Finger Lakes AVA at commercial harvest time points during the 2013 season. Approximately four kg of fruit was harvested for each cultivar from which a random sample of approximately 50 berries was used to express juice for pH, soluble solids, and titratable acidity. Titratable acidity was measured using a Titrino Plus 848 doser and 869 autosampler (Metrohm USA, Riverview, FL), soluble solids were determined by a digital pocket refractometer (Atago; Japan), and pH was analyzed using an Accumet Excel XL25 pH Meter (Thermo Fisher Scientific, Waltham, MA). From each harvested cultivar, 50 mL juice samples were manually pressed using cheesecloth, in triplicate, for protein quantitation. Harvested fruit that was not allocated to winemaking or juicing was kept frozen at -20 °C for further analyses.

GoFerm Protect, Fermaid K, and Lalvin ICV-GRE yeast were supplied from Lallemand (Santa Rosa, CA). Diammonium hydrogen phosphate (DAP) was purchased from Presque Isle

Wine Cellars (North East, PA) and potassium metabisulfite was sourced from Fisher Scientific (Waltham, MA). From each harvested sample, 750 g of berries were manually destemmed and weighed into 1 L fermenters, in triplicate. Berries were crushed manually and the resultant 0.75 liters of must was treated with 100 mg/L sulfur dioxide (added as potassium metabisulfite). Lalvin ICV-GRE yeast was rehydrated with GoFerm Protect as per the manufacturer's instructions, and pitched at an addition rate of 0.2 g/L. FermaidK and DAP were supplemented at rates of 0.25 g/kg and 0.3 g/kg respectively, 48 hours after pitching yeast. Samples were fermented on the skins at 18°C for seven days with daily punch downs. On day seven, residual sugar was determined to be below 0.5 g/L by Clinitest and wines were pressed manually using cheesecloth. Sulfur dioxide was added (60 mg/L) and wines were bottled in 187 mL glass bottles (Waterloo Container, Waterloo, NY) under N_2, and stored at 2 °C in the dark until needed for analyses at 6 months post bottling.

Condensed Tannin Quantitation in Grape Tissues and Experimental Wines

CT in 2013 experimental wines was measured by protein precipitation[30] and by phloroglucinolysis followed by HPLC,[34] as described elsewhere. To quantitate CT in grape berries, samples of 20 berries were taken from each harvested variety in triplicate. Whole berry samples were weighed, and skins and seeds were carefully separated using forceps and a scalpel. Skin and seed samples were weighed, and seeds were subsequently ground using a Genogrinder 2000 (Spex SamplePrep, Metuchen, NJ). CT was extracted from triplicate skin tissues and ground seeds via a 70% (v/v) acetone incubation and agitation on an orbital shaker overnight. A 2 mL aliquot of the extract was removed from each sample and acetone was removed under reduced pressure at 30 °C. Samples were brought up to a standard volume of 2 mL with filtered

water (Millipore Corporation, Billerica, MA). CT was then quantitated by protein precipitation using the method of Harbertson and colleagues.[30]

Evaluating CT Retention across Varietal Wines

A 2 mL sample of each experimental wine was centrifuged at 13,000g for 5 min before removing a 1 mL aliquot for the addition of seed CT extract in model wine. After 1 mL of each of the 33 wines was placed in a 1.5 mL microcentrifuge tube, 50 μL of the purified seed CT extract was added to each sample. Samples were briefly vortexed and incubated for 30 min at 20° C. Seed CT additions to a model wine control (12% (v/v) ethanol, 5 g/L tartaric acid, pH 3.3) was also evaluated in triplicate. After incubation, wine samples were centrifuged at 13,000g for 5 min. The supernatant was carefully removed by pouring, and CT in the pellet was quantified after resuspension and reading the absorbance @610nm before and after reaction with $FeCl_3$, similar to the Harbertson assay.[30]

Protein Quantitation in Juice and Wines by SDS-PAGE

Chilled juice and experimental wine samples were clarified by centrifugation and 10 mL was added to a 50 mL centrifuge tube. Proteins were precipitated by slow addition of 40 mL of a saturated ammonium sulfate solution to the samples at 2 °C. Solutions were left at 2 °C overnight and centrifuged the next morning at 12,000g for 30 min. at 4°C. The supernatant was decanted and the protein pellets were resuspended in filtered water (Millipore Corporation, Billerica, MA). Samples were desalted against filtered water using Slide-A-Lyzer MINI dialysis devices (Thermo Scientific, Waltham, MA) with a 3.5 kDa molecular weight cut off and lyophilized.

Lyophilized samples were reconstituted in either 0.25 mL or 1mL filtered water before quantitation by SDS-PAGE and densitometry. Twenty μL of this protein solution was mixed 1:2

with sample loading buffer, briefly vortexed, heated in a 95 °C water bath for 5 min, then set on ice. Buffered protein solutions were then centrifuged at 10,000g for 1 min to recover droplets suspended on the upper portions of the tubes. Twenty µL of each buffered protein solution (representing 12.5- 100 µL of the original wine) was then loaded onto a Mini-PROTEAN TGX Precast 12% glycine gel. *E.coli* lysate in the amounts of 0.625, 3.125, and 6.250 µg (GenLysate; G-Biosciences, St. Louis, MO), and Mark12 Unstained Standard (Life Technologies, Carlsbad, CA) were included in each run. Electrophoresis was performed as previously described.

Gels were stained overnight in SYPRO Ruby protein gel stain (Bio-Rad Laboratories Inc., Hercules, CA), as this stain is reported to give a dynamic range of three orders of magnitude and reproducibility of <10%.[92] Stained gels were fixed in a 10% (v/v) methanol, 7% (v/v) acetic acid solution for 60 min, as per the manufacturer's instructions.

Gels were rinsed with filtered water before scanning on a Typhoon 9400 gel scanner (GE Healthcare, Cleveland, OH). Proteins in juice or wine were quantitated against the GenLysate standard curve set on each gel (ImageQuant TL, GE Healthcare Life Sciences, Pittsburgh, PA).

In-Gel Trypsin Digests for Multiple Reaction Monitoring (MRM)-based Quantitation

Wine proteins for the 11 grape varieties were first isolated and separated by SDS-PAGE, as described above. Bands were excised, sliced into 1 mm sections, and transferred to microcentrifuge tubes for in-gel digests, as described in Supporting Information.

Selection of Peptides for Multiple Reaction Monitoring (MRM)-based Quantitation

From the database search results, two unique peptides with an acceptable extensive homology score ($p<0.05$) from each protein of interest were selected for MRM-based quantitation. Peptide charge and *m/z* was verified by manual inspection of raw ER scan data and specified as the Q1 mass. For each peptide, three product ions were selected based on *b* or *y*

MS/MS fragment ions predicted by the Mascot database search and confirmed by manual inspection of raw CID data as Q3 masses. In cases where the target peptide was detected in significant amounts with multiple charge states, each charge state and appropriate Q1 and Q3 masses were included in the MRM acquisition method.

Wine Protein Quantitation by Nano-LC/MS/MS

A trypsin digested exogenous protein, apomyoglobin, was added post digestion at equal concentration to all samples as an internal standard. Two peptides were selected with three transition ion pairs specified for each peptide for inclusion in the MRM acquisition method. Each lyophilized in-gel digest was reconstituted to 10 ng/µL in 2% acetonitrile (ACN)/0.5% formic acid (FA) containing 5 fmol/ µL apomyglobin digest. Ten µL was injected via autosampler onto an Acclaim PepMap 100 nanoViper C18, 100 µm x 2 cm, 5 µm, 100 Å trapping column (Thermo Scientific, Hanover Park, IL USA) at 20 µL/min for 5 min with 2% ACN/0.1% FA as the trapping solvent. The peptides were separated on a 75 µm x 15 cm, 3 µm, 100 Å C18 (CMP Scientific, East Newark, NJ USA) column using a linear, 45 min gradient under the following parameters: Solvent A: 0.1% FA, Solvent B: 95% ACN/0.1% FA, Flow rate: 300 nl/min, Column temperature: 30 °C, Gradient: 0-3-5-45-46-52-53-73 min., 5-5-10-32-90-90-5-5 %B. The MS data acquisition began after 4 min using Analyst ver. 1.6.1 (AB Sciex) on a 4000 Q Trap (AB Sciex) equipped with a MicroIonSpray II ion source. The acquired raw MRM data was processed using MultiQuant ver 2.1.1 (AB Sciex). Each extracted transition ion pair was smoothed using a 3 point Gaussian width and integrated using software defaults. A single apomyoglobin transition ion pair was selected as the internal standard for all other MRM transitions. For comparison, a wine produced from *V. riparia* was chosen as the standard and

each transition was assigned an arbitrary value of 100, with a linear through zero curve fit, to obtain relative concentration values for the other samples which were treated as "unknowns".

Modeling CT Adsorption to Protein

The adsorption of CT to proteins was modeled by the linear form of the Freundlich equation. Equation 3.1:

$$log(q_s) = \log K_F + b_F \log [C_S]$$

Where q_s is the mass ratio (mg/mg) of CT adsorbed to the amount of protein in a volume of wine, C_s is the equilibrium concentration of CT left after fining (mg/L), K_F (L/mg) is an empirical constant that indicates the adsorption capacity of the fining agent (protein), and b_F is dimensionless and represents the adsorption intensity. A plot of log (q_s) vs log [C_s] was constructed using JMP Pro 11 (by SAS, Cary, NC), and K_F and b_F determined from the intercept and slope, respectively.

Statistical Analysis

All statistical testing and modeling for this study was performed using JMP Pro 11 (by SAS, Cary, NC), with a Type 1 error rate set to 0.05. One-way ANOVA and Tukey's post hoc HSD was used to evaluate for differences among the wine variety classifications (native *Vitis*, interspecific hybrids, and *V. vinifera*) in wine CT by protein precipitation, wine CT by HPLC, skin CT, seed CT, total CT in fruit by weight, wine protein, juice protein, and CT precipitated after addition to experimental wines. The effect of wine variety class (native *Vitis*, interspecific hybrids, and *V. vinifera)* on b_F, the slope of the log-log plot, was also modeled using an interaction term between "type" and log C_s.

RESULTS AND DISCUSSION

Preliminary Observations– Protein Identification and Compositional Analysis of Pellets Formed following Purified Seed CT Addition

Our previous work observed that low CT concentrations in wines produced from interspecific hybrids as compared to *V. vinifera* were explained not only by lower CT concentration in the former, but also by the greater CT binding of their cell wall material.[91] We thus hypothesized that post-fermentation CT additions in the absence of cell wall material should be equally effective in increasing CT in both interspecific and hybrid wines. However, following addition of a purified and fully solubilized seed CT extract produced from pooling multiple grape varieties (mDP = 6.55, mole fraction of gallolyation = 0.126) to Baco noir, Maréchal Foch, Corot noir, DeChaunac, Cabernet Franc, Merlot, Cabernet Sauvignon, and Pinot noir wines from 2012, we observed immediate formation of a precipitate. This result could explain the incomplete recoveries of CT additions observed by other authors.[35, 36, 93] These previous studies did not mention the formation of a precipitate, but it may have been overlooked because the initial CT preparations were not purified and were initially turbid. Following isolation of a pellet, we observed several Coomassie Blue staining bands on an SDS-PAGE gel, which were subsequently excised from the Corot noir lane and identified by nanoLC-MS-MS as PR proteins (Table 3.1).

TABLE 3.1 Identity of Major Proteins Isolated from Precipitate in Red Wine

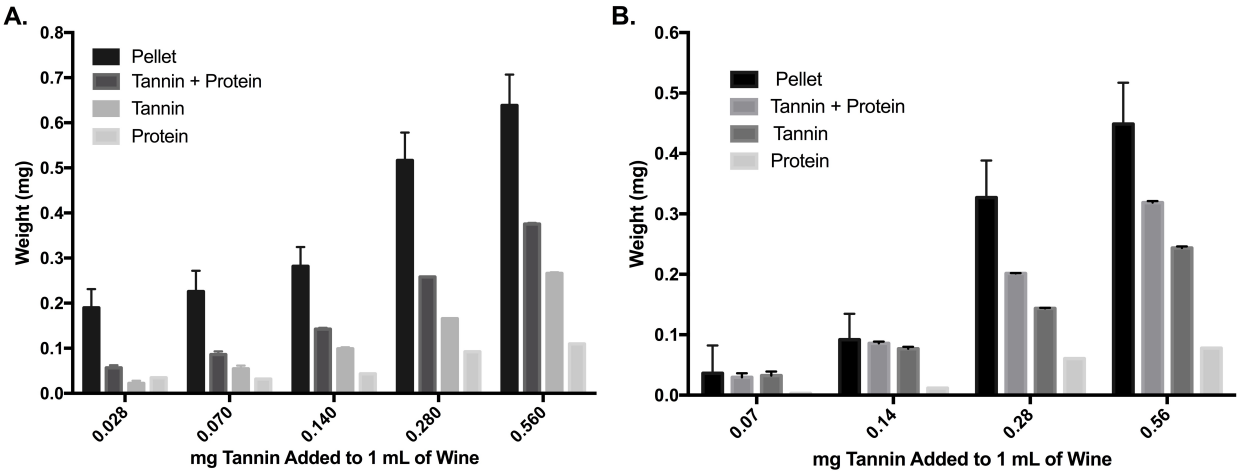
Band	Identification	Score[1]	Mass (Da)	pI
1	glucan endo-1,3-beta-glucosidase [Vitis vinifera] (gi|225441373)	5947	36711	8.45
2	class IV chitinase precursor [Vitis vinifera] (gi|33329392)	1737	28366	5.38
3	VVTL1 precursor [Vitis vinifera] (gi|526117633)	3343	24947	4.94

[1] Protein score is based on the ion scores from the mass spectra that can be matched to the amino acid sequence in the protein, with higher scores indicating higher confidence in the match.

Although PR proteins have been studied extensively in white wines for their contribution to haze,[94] this class of proteins has not been examined extensively in red wines as they are thought to be largely removed by CT extracted during fermentation.[45]

To confirm that protein was a major component of the precipitate formed following CT addition to wine, Maréchal Foch grapes were vinified under standard conditions, and the pooled seed CT extract described above added to the finished wine at increasing amounts. The total pellet mass, CT content, and crude protein content for each seed CT level addition are shown in Figure 3.1A. Initial addition of a small amount of CT (0.028 mg in 1 mL of wine) resulted in a precipitate with relatively low protein and CT content (29% w/w, Figure 3.1A). This initial precipitate may have included other non-proteinaceous compounds capable of forming aggregates with CT, e.g. grape-derived polysaccharides like RGII.[95] Figure 3.1B displays the pellet weight and protein and CT content of the pellet after subtracting this initial precipitation event observed in the lowest CT addition (0.028 mg). With further CT additions, both the protein and CT content of the precipitate increased ($p < 0.05$), the sum of protein and CT accounted for 60-90% of the pellet weight, and for the highest CT additions (0.28 and 0.56 mg/mL), protein accounted for ~20% of the pellet weight (Figure 3.1B).

FIGURE 3.1 Wine Precipitate Composition- Condensed Tannin and Protein



A. Total pellet mass, CT content, and protein content of precipitates from Maréchal Foch wine following seed CT additions.

B. Adjusted total pellet mass, CT content, and protein content by seed CT addition. Adjusted values were calculated as the pellet mass, CT or protein for a given CT addition (0.07 to 0.56 mg) minus the total pellet mass, CT, or protein observed with the smallest seed CT addition (0.028 mg)

Since the Maréchal Foch wine used had 198 mg/L protein (about 0.02% protein by weight) as determined by SDS-PAGE, the 20% w/w protein concentration in the pellet would represent nearly a 1000-fold enrichment over its concentration in wine. These results are comparable to work by Waters et. al, who reported that ~22% of the weight of precipitate in red wine bottles could be attributed to proteinaceous materials.[96] This group also reported that different bottle deposits can have differing proportions of protein, anthocyanins and CT, and that uronic acid (from pectin) was not found in red wine precipitates.[96]

The crude protein weight is calculated by mg/g N x 6.25, and thus the weight of any associated glycosylations on these proteins are not accounted for in this data. Numerous publications have shown that many juice and wine proteins, including some PR proteins, are glycosylated,[97, 98] and their concentration would be underestimated using the standard approach to calculating crude proteins. It is also possible that pre-formed protein-polyphenol complexes

existed in the wine,[99] which could have contributed non-CT polyphenols to the precipitate. Waters and colleagues proposed this type of covalent protein-polyphenol interaction in red wine bottle deposits.[96] Other potential contributors to the precipitate that were not evaluated include potassium bitartrate, as removal of macromolecules prevents their inhibition potassium bitartrate precipitation.[100] Regardless, protein appeared to be a major non-CT component of the precipitate following typical rates of CT addition (140-560 mg/L), especially since the response from glycoproteins like PR proteins would be underestimated in measurements of total nitrogen.

Condensed Tannin and Protein in 2013 Fruit, Juice, and Experimental Wines

To evaluate if juice and wine protein content explained variation in CT retention among finished wines, 11 different grapes varieties representing three major classes (*V. vinifera*, native *Vitis* spp. and interspecific hybrids) were sourced from the Finger Lakes region of NY State and vinified under standard conditions. Data on grape sources and fruit chemistry, including vineyard location, soluble solids, titratable acidity, pH, and berry weights, can be found in Supplementary Table S3.1 (Appendices).

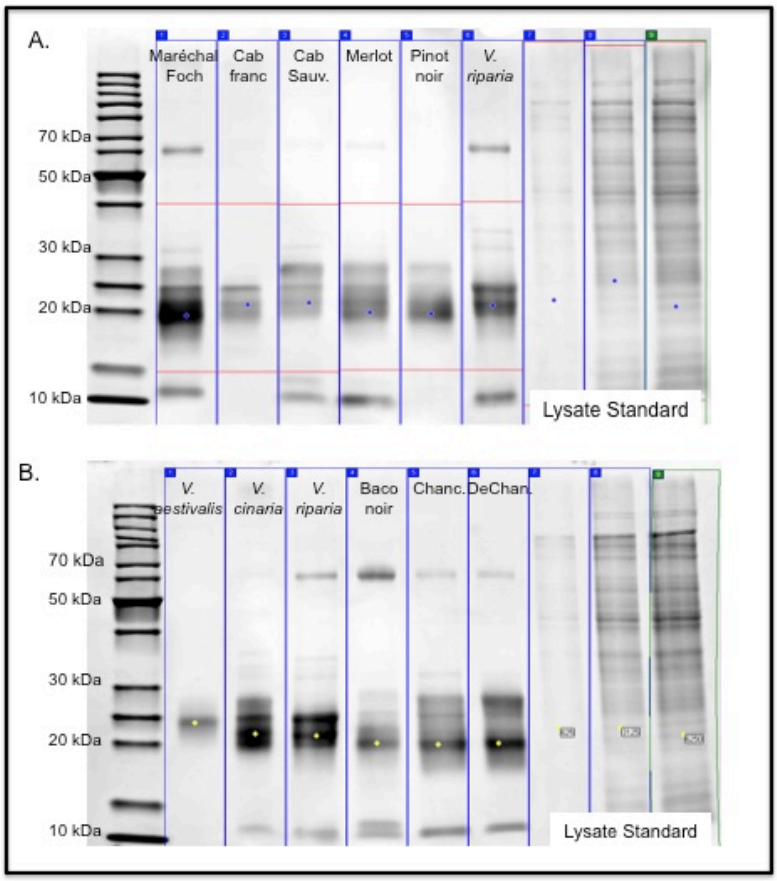
Data on skin, seed, and wine CT are shown in Supplementary Table S3.3 (Appendices). In agreement with our previous work,[91] *V. vinifera* samples contained significantly more CT in skins (0.660 vs 0.149 mg/g) and seed tissues (2.30 vs 1.00 mg/g), compared with interspecific hybrid grapes. Native *Vitis* spp. had similar amounts of skin CT to interspecific hybrids and significantly more seed CT (1.60 vs 1.0 mg/g) by berry weight. Total CT in the native *Vitis* species was about 60% that of *V. vinifera*, considerably more than the 100-fold lower concentrations in native *Vitis* in another recent report.[101] A potential explanation for this discrepancy is that the other report used methanol rather than 70% acetone to perform

extractions, which would not be a sufficiently strong solvent to disrupt CT:protein interactions.[102]

Because the concentration of CT in wines produced from interspecific and native *Vitis* was below the detection limit of the protein precipitation assay (50 mg/L), it was necessary to quantitate wine CT by an alternate method (phloroglucinolysis followed by HPLC) and convert to protein precipitation equivalents. CT by the HPLC and protein precipitation methods was well correlated in *V. vinifera* wines (Supplementary Figure 3.1, Appendices) with a slope of 1.13 and an r^2 =0.93, comparable to values of 1.15 and 0.91 in previous work.[20] In agreement with our previous findings, wines produced from *V. vinifera* had significantly more CT than those produced from interspecific hybrids as measured by HPLC-phloroglucinolysis (189.7 vs 17.4 mg/L CE, see Supplementary Table S3.3, Appendices). Wine CT from native *Vitis* spp. (25.9 mg/L) was also significantly less than that of *V. vinifera* samples (189.7 mg/L), and not significantly different from wines produced from interspecific hybrids. In contrast to some other studies,[30, 59] we observed a modest correlation between total CT in fruit (skins + seeds) and wine CT concentrations, and a better correlation with skin CT and wine CT (Spearman's coefficients 0.815 and 0.924, respectively). However, this evaluation was complicated by the fact that only 3 of the 11 varieties in this study produced wines having >100 mg/L CT (Pinot noir, Cabernet Sauvignon, and Merlot), while most other wines had negligible wine CT (5-40 mg/L).

Protein content in juice and wines as determined by SDS-PAGE densitometry is shown in Supplementary Table S3.3, Appendices. Representative SDS-PAGE images of proteins isolated from eleven varieties are shown in Figure 3.2A and 3.2B. The major bands on the gel correspond to the proteins identified by nanoLC-MS/MS (Table 3.1).

FIGURE 3.2 Representative SDS-PAGE Gels used in Quantitation of Individual or Total Proteins



The section between 20 kDa and 40 kDa (as depicted between the boxed in area of gel 2A) was excised for quantification of specific proteins given in Table 1 by a multiple reaction monitoring method. A common *V.riparia* sample was added to both gel 2A (lane 6) and gel 2B (lane 3) as to control for inter-gel variance. For total protein quantification, the entire lane was integrated. The far left lane shows a protein standard ladder, and the far right three lanes are standard additions of E. coli cell lysate for quantitation by densitometry.

The minor bands at 12 kDa and 65 kDa were not identified, but possibly correspond to two other PR proteins (invertases and lipid transfer protein) identified in other studies.[94]

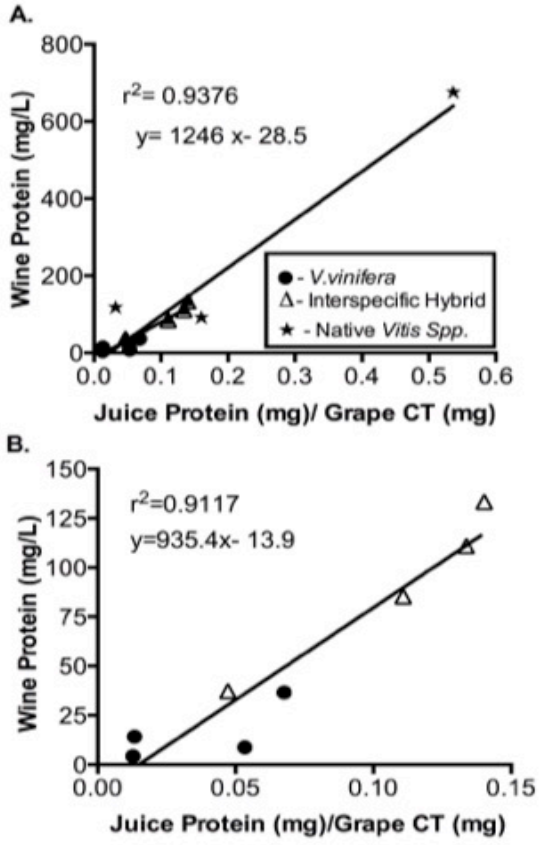
Disease pressure in the vineyard has been cited to alter the levels of proteins in juice and wines,[103] but all samples appeared disease-free at time of harvest. Vines experiencing lower UV exposure have also been reported to yield higher juice proteins levels compared to controls,[104] but these vineyard parameters were not evaluated in this study. Juices produced from native *Vitis* grapes had significantly more protein (avg. 705.7 mg/L) than either interspecific hybrid

(avg. 175.8 mg/L) or *V. vinifera* juices (146.2 mg/L). Pocock et al. reported that PR proteins are the dominant class of proteins represented in free-run juice, with thaumitin-like proteins and chitinases the major proteins in hand pressed juice.[105] The extraction of the additional proteins into juice was observed with mechanical harvesting and increased transport times, which allowed for longer contact times on broken grape skins to enhance extraction.[105] These authors reported total protein concentrations of 51 mg/L and 203 mg/L for hand pressed Pinot noir and Sauvignon blanc, respectively, which is in agreement with the range we report here for *V. vinifera* (44-254 mg/L). Data on juice protein concentrations in interspecific hybrids and native *Vitis* spp. are lacking from the literature, but speculatively the high concentrations of proteins in the native *Vitis* could be related to their greater disease resistance. One group has pointed out that efforts to select for varieties with enhanced PR protein expression and disease resistance could diminish wine quality in the context of white wine haze,[106] but not in the context of red wine CT content and mouthfeel.

Similar to juices, wines produced from native *Vitis* spp. averaged 3.2 times more protein on average than those produced from interspecific hybrid varieties (295.5 vs. 91.7 mg/L) and 18.5 times more protein than wines produced from *V. vinifera* (16.0 mg/L). Previous reports using SDS-PAGE have also reported that the major proteins in red wines produced from *V. vinifera* and some interspecific hybrids are PR.[107] However, SDS-PAGE generally does not detect yeast mannoproteins because the large carbohydrate moieties on these molecules (up to 90% by weight), can exclude them from entering the gel.[108] This may explain why our wine protein values for *V. vinifera*, and particularly Pinot noir (avg. 8.7 mg/L), fell below the range (50-102 mg/L) reported by Smith and colleagues for a large survey of Pinot noir wines using a trichloroacetic acid/acetone precipitation followed by staining.[109] However, mannoproteins are

yeast-derived and were thus not expected to explain differences in CT retention among grape varieties. Furthermore, mannoproteins also are reported to stabilize CT in solution rather than form precipitates.[95] Thus, we believe our SDS-PAGE based quantitation method to be more useful in our particular application as compared to the colorimetric method of Smith.[109]

FIGURE 3.3 The Correlation of Wine Protein to the Juice Protein: Grape Condensed Tannin Ratio



A. Linear regression of wine protein vs. the ratio of juice protein to grape CT for all 11 grape varieties, and B. regression based only on 8 *vinifera* and interspecific hybrids (native *Vitis* excluded) cultivars studied. Error bars represent standard deviations across winemaking replicates (n=3).

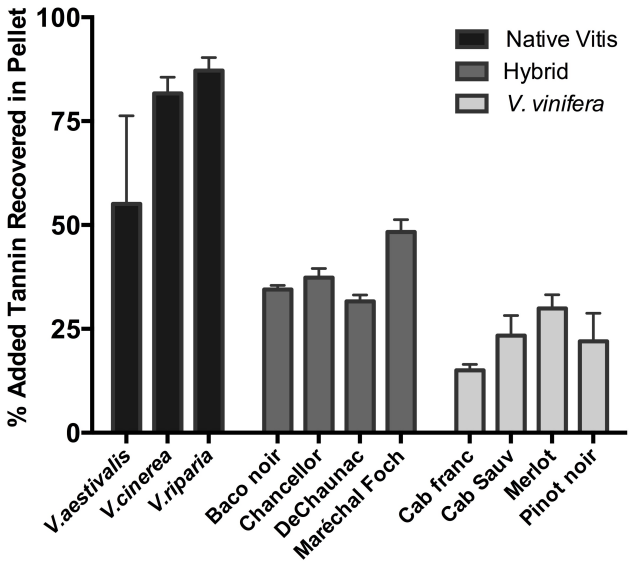
The concentration of wine protein was less than juice protein for all varieties, but the ratio of wine-to-juice protein varied from less than 10% to over 80% (Supplementary Table S3.3, Appendices).

To evaluate if this variation in protein extraction could be related to binding of protein to grape CT during fermentation, wine protein was modeled as a function of the juice protein:CT ratio (Figure 3.3). Highly linear models could be generated using data from all 11 cultivars (r^2 = 0.93, Figure 3.3A) and when eliminating the high-protein native *Vitis* spp. cultivars (r^2= 0.91, Figure 3.3B) from the model. The slopes of the two models were not significantly different (p = 0.33, two-tailed t-test), indicating that the model was robust across a range of juice protein and berry CT concentrations. Models based on individual CT components (skin CT, seed CT) did not result in improved models (data not shown).

Condensed Tannin Retention in Experimental Wines

To evaluate if exogenous CT retention to wine was limited by wine protein, purified seed CT extract (150 mg/L) was added to the experimental wines (33 wines produced from 11 grape varieties) described in the previous section.

FIGURE 3.4 Precipitated Condensed Tannin From Wines After Seed Tannin Addition

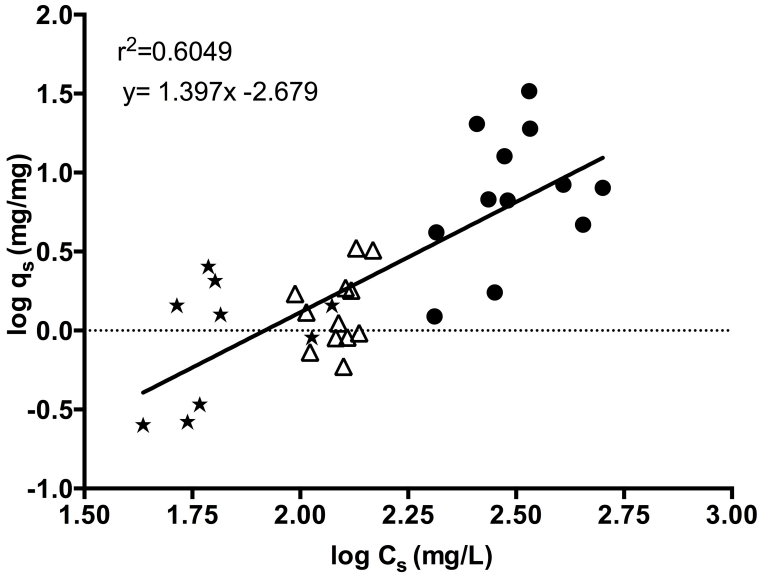


Tannin precipitated (expressed as % of tannin added) from varietal wines after a 150 mg/L addition of seed tannin. Error bars represent standard deviations for winemaking replicates (n=3).

The percentage of the CT addition lost was quantitated in pelleted material and is depicted in Figure 3.4. Wines produced from native *Vitis* spp. precipitated 3.3 times more CT than *V. vinifera* wines and 2 times more than wines produced from interspecific hybrid grapes. This corresponds to an average CT retention of 25% for native *Vitis*, 62% for interspecific hybrids, and 77% for *V. vinifera* wines after a short (30 minute) incubation. Further time points were not investigated, but the reaction of purified CT with proteins is reported to complete within 15 min.[110]

Protein-CT interactions do not show Langmuir type saturation behavior, but instead are better modeled by the Freundlich equation. As shown in Figure 3.5, the linearized Freundlich model accounted for 60.5% of the variation in the ratio of precipitated CT: total protein ($\log q_s$). Including "type" (native *Vitis* spp. vs. interspecific hybrid vs. *V. vinifera*) in the model showed that this factor did not have a significant effect ($p=0.31$).

FIGURE 3.5 Linearized Freundlich Isotherm-Protein Fining and Equilibrium Condensed Tannin in Wine



Linearized Freundlich adsorption isotherm at 22°C. The log transformation of equilibrium wine CT (Cs) is plotted on the x-axis, vs. the log transformation of the mass ratio (qs) of CT adsorbed: the amount of protein in wine, on the y-axis.

Additionally, the log C_S (log equilibrium CT) by type interaction term was non-significant ($p=0.98$). This demonstrates that the model was not significantly influenced by grape types. The model produced a significant, positive, relationship between log C_S and the amount of CT adsorbed at a given level of wine protein (log q_s, $p<0.001$), supporting our hypothesis that protein adsorption limits CT retention. Previous studies have shown that the formation of insoluble CT-protein complexes is influenced by protein, CT, and polysaccharide concentrations and molecular structure, in addition to alcohol, pH and ionic strength,[102, 111] which may account for the unexplained variation in the Freundlich model. In particular, grape-derived polysaccharides such as arabinogalactans are expected to reduce the amount of insoluble complex formation and thus reduce precipitation, but these were not quantitated. Additionally,

several studies have demonstrated that an excess of protein in relation to CT leads to shift from the formation of insoluble to soluble CT-protein complexes, explaining the less efficient removal of CT from higher wine protein cultivars.[110, 112]

The value of b_F (slope) in the best fit model is >1, indicating strong sorption of CT by the protein. Authors that have previously characterized these interactions in more strictly controlled experiments have seen similar results.[4, 111, 113] Considering that many parameters that should affect CT:protein interactions were not controlled across these experimental wines, e.g. polysaccharides, protein type, initial CT composition, pH, etc.), the robustness of the Freundlich model is impressive, and strengthens the hypothesis that wine protein limits the effectiveness of exogenous CT additions. Lower CT retention is expected in cases of high wine protein, which could arise from either high juice protein or low berry CT, or a combination of the two. These conditions describe interspecific hybrid varieties, which may explain previous reports of minimal effects of CT additions on wines produced from these grapes.[34] Grape protein binding of CT may also help explain why other literature reports show incomplete recovery of CT following addition to wine, e.g. no significant increase in CT was observed following a 200 mg/L commercial seed CT addition to Shiraz.[35]

Relative Quantitation of Specific Pathogenesis-Related Proteins

We then hypothesized that PR proteins may vary in their CT-binding ability and that specific fractions may be responsible for the majority of CT adsorption and loss. To evaluate this hypothesis, three different proteins identified from CT-protein complexes in initial work on Corot noir and two from Maréchal Foch in the MRM method validation phase were quantitated in 11 wines (one from each cultivar) by nanoLC-MS-MS (Supplementary Table S3.2, Appendices). *Vitis riparia* wine was used as a reference sample and assigned a value of 100 for

all proteins of interest, and relative concentrations of proteins in other wines are reported with the respect to this reference (Supplementary Table S3.4, Appendices). Considerable differences in relative concentrations were observed across types. Wines produced from interspecific hybrid cultivars had 3.7-fold higher VVTL1 precursor than wines produced from native *Vitis* and over 20-fold more than *V. vinifera*. Wines produced from interspecific hybrid grapes also had 2-fold more thaumatin-like protein than wines produced from native *Vitis* and 6.3-fold more than wines produced from *V. vinifera*.

However, this additional data on relative differences in specific wine proteins was not useful in improving models of CT losses following exogenous CT addition. Only relative quantities of Peroxidase 4 in the 11 wine samples was a significant predictor of precipitated CT ($p=0.0009$, $r^2= 0.7232$) and log Cs ($p=0.0269$, $r^2= 0.4364$) – other relationships were insignificant, either alone or in combination (data not shown). The correlation between the peroxidase and CT loss may have been an artifact of the good correlation between peroxidase and total protein ($p= 0.0002$, $r^2= 0.7989$). Thus, although quantitative differences of these specific proteins by grape type and cultivar were apparent, overall total protein by densitometry was more useful in explaining differences in CT retention in wines. Potentially, changes in protein structure such as unfolding would impact CT-protein interactions. Previous studies have shown that loosely configured proteins have a much higher affinity for CT than tightly structured globular proteins,[4] but this level of detail was not assessed in this study. In summary, we have demonstrated that retention of exogenous CT added to finished wine is inversely correlated with the concentration of wine protein, and that the binding is well modeled by a Freundlich isotherm across many types of grapes. The co-precipitation of CT with protein explains the higher protein to CT ratios observed in interspecific hybrid and native *Vitis* species, in comparison with *V.vinifera* wines.

The primary proteins responsible for this binding appear to be PR proteins – while the role of these proteins in wine haze is well established,[94] their role in limiting the retention of CT after post-fermentation additions has not been reported. This work suggests that processing techniques to remove proteins from red wines, e.g. bentonite additions, before or after fermentation may facilitate later CT additions, and is subject of current investigation in our lab. Future evaluations will also investigate whether juice protein can help explain variation in CT extraction from grapes during fermentation.

CHAPTER 4

RELATIONSHIP OF SOLUBLE GRAPE-DERIVED PROTEINS TO CONDENSED TANNIN EXRACTABILITY DURING RED WINE FERMENTATION[c]

INTRODUCTION

Recent research has shown that CT content is correlated with both red wine price point and consumer perception of red wine overall quality.[24] The best known organoleptic effect of CT is to increase wine astringency by removing lubricating salivary proteins of the oral cavity and/or by direct interaction with oral mucosal proteins.[18] Consequentially, the overall astringency of a wine is well-modeled by protein precipitation assays.[20] CT can also participate in reactions with anthocyanins and other wine components to yield stable polymeric pigments, as well as react with oxidation products,[11] which may further explain their correlation with wine quality.

While they are typically at negligible concentrations in juice following crushing, CT are extracted into must during fermentation through contact with skins and seeds. CTs in ripe grape skins are found in intercellular vacuoles or associated with apoplastic cell-wall material, both of which require cell rupture to permit release during alcoholic fermentation.[114] Skin CT extraction generally begins after alcoholic fermentation, commencing and plateauing prior to fermentation completion.[26] Extraction of CT from seeds is slower, and shows a lag phase as the outer seed coat hydrates before increasing at a near-linear rate through the end of fermentation.[115] Thus, the degree of CT extraction and the final CT concentration can be manipulated to some extent

[c] Reprinted with permission from: Springer, L. F.; Chen, L.; Stahlecker, A. C.; Cousins, P.; Sacks, G. L., Relationship of Soluble Grape-Derived Proteins to Condensed Tannin Extractability during Red Wine Fermentation. *J. Agric. Food Chem.* **2016**. Copyright 2016 American Chemical Society

through altering the skin and seed contact time, degree of maceration (e.g. pumpover frequency) and fermentation temperature, as highlighted by several recent reviews.[11, 114]

However, even in cases where winemaking conditions are constant, considerable variation can be observed in tannin extractability across grape samples.[91] One report observed that 4.9 to 61% of tannin is extracted under controlled fermentation conditions,[30] while others have shown poor correlations between total grape CT and final wine CT.[91] Low extractability is particularly notable in the case interspecific hybrid cultivars, which typically show < 5% extraction.[91] Potentially, variation in CT extraction could be explained by tannin location within the berry, although grape skin CT only shows a slight improvement as compared to total grape CT in predicting final wine CT across multiple cultivars.[91] The wide variability in CT extraction is in contrast to other grape-derived phenolics like anthocyanins, which show high correlations between grape and wine under identical fermentation conditions.[31]

Several hypotheses have been advanced to explain variation in CT extraction. Singleton and Trousdale observed that adding anthocyanin to fermenting white wine increased CT extraction,[45] potentially because of the enhanced solubility of reaction products of anthocyanins and CT. Anthocyanin concentration has been proposed as an explanation of CT extractability variation among red grapes during maturation.[46, 47] Kilmister and coworkers reported CT extraction was also greater in Syrah lots with higher grape anthocyanin content, although the ratio of anthocyanins and CT in fruit was not reflected in the wine.[87] As an alternative to polymeric pigment formation, the authors proposed that enhanced CT extraction was a consequence of anthocyanin competition for binding sites on cell walls.[87, 116] In contradiction to this hypothesis, interspecific hybrid grapes demonstrate very low tannin extractability, yet usually have higher anthocyanin concentrations than *V. vinifera.* [91, 117]

CTs will bind to pomace even once they are extracted (or added exogenously), and variation in grape cell wall quantity, composition, or structure has been proposed to explain variation in CT extraction.[40] CTs interact with cell wall polysaccharides through hydrogen bonding and hydrophobic forces,[40] with a high affinity for pectin, and lesser affinities for other cell wall carbohydrates like xyloglucan, starch and cellulose.[41] Pectin can also confer enhanced flexibility to cell wall material, exposing more CT binding sites and surface area for interaction[41,43]. Bindon and colleagues proposed that the increase of cell wall porosity during ripening facilitates its retention of CT in cell wall material, reducing extraction into wine and modulating the molecular size of CT in wine.[43] In this respect, ripening-related changes to cell wall composition and structure, such as changes in pectin-methylation and solubilization, and the loosening of the xyloglucan-cellulose network, can influence the release and/or adsorption of phenolic species.[44]

CT extraction may also be limited by grape proteins rather than (or in addition to) grape polysaccharides. CT binding capacity of cell wall materials sourced from 12 *V. vinifera* and interspecific hybrid cultivars was best correlated with crude protein content of skin cell wall material, while a much weaker correlation was observed with pectin.[91] In more recent work, we demonstrated that retention of CT added to finished wines could be well-modeled from initial wine protein and wine tannin concentrations.[118] These CT-binding proteins were subsequently identified as grape-derived PR proteins,[118] which have been well studied in white wines due to their role as haze-causing nuisance proteins.[94] PR proteins were significantly higher in wild *Vitis* and interspecific hybrid grapes than in *vinifera,*[118] in agreement with winemaker observations of greater proteinaceous foaming during fermentations of cold-hardy hybrids.[119] The ability of CT to bind protein is well documented,[94, 113] but the role of grape proteins in limiting retention of exogenous CT had not been described in the literature. In previous work, low CT extraction

during fermentation was hypothesized to result from high grape protein content,[118] but this hypothesis could not be tested due to the very low CT concentrations in the finished wines.

The first goal of the current study was to determine if CT in red wines produced from different cultivars under identical fermentation conditions could be better modeled from initial grape protein and grape CT than by grape CT alone. The second goal was to determine if decreasing must protein content prior to fermentation could increase tannin extractability and final wine tannin.

MATERIALS AND METHODS

Chemical Reagents

Acetone, albumin from bovine serum, (+)- catechin hydrate, ethanol (reagent grade), iron (III) chloride, methanol, sodium dodecyl sulfate (SDS), triethanolamine (TEA), trichloroacetic acid (TCA), and Tris-HCl were obtained from Sigma Aldrich (St. Louis, MO) at the highest available purity unless otherwise specified. Acetic acid, Clinitest® tablets (Bayer, Pittsburgh, PA), potassium metabisulfite, L-(+)-tartaric acid, sodium hydroxide, and were sourced from Fisher Scientific (Waltham, MA). Sodium Bentonite (Volclay KWK Krystal Klear) was obtained from Presque Isle Wine Cellars (North East, PA). Amido Black was purchased from Spectrum Chemical MFG Corp (New Brunswick, NJ).

Grape Samples

For Experiment 1, assessing the impact of grape juice protein on CT extraction, fruit was harvested from both CA and NY vineyards in 2014. DeChaunac, Maréchal Foch, Regent, Cabernet Sauvignon, Cabernet franc, and Lemberger grapes were harvested from research vineyards in Solano County, CA, and Dornfelder was harvested from a research vineyard in Madera County, CA. Samples were shipped on ice to the NYS Agricultural Experiment Station

(Geneva, NY). Cabernet Sauvignon, Cabernet franc, Lemberger, and Maréchal Foch were harvested from commercial vineyards in the Finger Lakes region of NY. Rougeon, Vincent, Baco noir, Chancellor, and DeChaunac were harvested from the USDA – Cold Hardy Grape Collection (Geneva, NY). All samples were kept frozen at -20°C for *ca.* six months until needed for winemaking and subsequent analyses.

For Experiment 2, assessing the impact of protein removal on CT extractability, Lemberger and Maréchal Foch were hand harvested from the Finger Lakes region of NY during the 2015 harvest season, from the same vineyards as Experiment 1. Fresh harvested grapes were taken to the lab for immediate processing.

Winemaking

For Experiment 1, frozen grapes (~150 g) were weighed into triplicate fermenters, defrosted at 4 °C, and manually crushed. The musts were transferred to 250 mL glass jars and treated with 100 mg/L sulfur dioxide (added as potassium metabisulfite). Musts were left overnight at 18 °C to thaw and Lalvin ICV-GRE yeast was added (0.2 g/L) after rehydration with GoFerm Protect, as per the manufacturer's instructions. Solvent jar lids were drilled and fitted with airlocks to allow for CO_2 escape during fermentation, while preventing oxygen ingression. Fermentations were carried out over 9 days until dryness was achieved, as determine by Clinitest tablets. Jars were swirled daily to submerge the cap before manually pressing through cheesecloth. The volume of pressed wine was recorded and 60 mg/L sulfur dioxide was added before bottling in 100 mL solvent jars. Wines were stored for one week at 4 °C post bottling before CT quantification.

For Experiment 2, each of the two varieties (Lemberger and Maréchal Foch) was divided among one of five groups in triplicate (four protein removal treatments and one control group,

fifteen total fermentations per variety). For each fermentation, 750 g of berries were manually destemmed, weighed into a 1 L glass jar, and manually crushed. A 100 mg/L sulfur dioxide was added as potassium metabisulfite and musts were left at 4 °C overnight. The following day, musts were manually pressed through cheesecloth to yield *ca.* 500 mL juice per replicate. Skins and seeds were saved for later recombination with the juice, as described below for all treatments and the control. A juice aliquot (2 mL) was saved as a "before treatment" sample for each replicate. Juice treatments were as follows:

i) Control- pressed juice was kept separate from skins and seeds at room temperature (20 °C) for about 2 hours, analogous to the time required for other juice treatments.

ii) Heating- juices were heated in an Erlenmeyer flask (95 °C, 3 min) using a heating plate and stirbar. After cooling to 20 °C, juices were filtered through cheesecloth before being added back to skins and seeds in the fermenters.

iii) Flash freezing- juices were rapidly frozen using dry ice and subsequently thawed at room temperature before adding back to skins and seeds.

iv) Tannin addition- a commercial grape tannin preparation (BioTan; Laffort) was added to juices at a rate of 600 mg/L, and thoroughly mixed for an hour using a stir bar and Erlenmeyer flask.

v) Bentonite- a 6% slurry of bentonite was added to juices in an Erlenmeyer flask and stirred for one hour. The bentonite was settled by centrifuging (5000g, 10 min).

Following each treatment, a 2 mL sample of juice was collected and stored at -20 °C for later analysis ("after treatment" sample). The remainder of the treated (or control) juices were added

back to the fermenter which contained the corresponding grape solids (skins and seeds) that they were pressed from. The winemaking protocol was identical to Experiment 1, and must samples (2 mL) were taken daily during the course of fermentation (Days 1-9). Wines were pressed and bottled in 187 mL bottles with crown caps after Day 9.

Juice Protein Quantitation

Juice protein was quantified in both Experiments 1 and 2 by adapting a previously described method based on Amido Black staining,[120] with a minor modification. Instead of spotting prepared juice samples on a large nitrocellulose filter and excising individual sample spots, individual nitrocellulose filtration units were assembled using 13 mm nitrocellulose filters (Sigma Aldrich, St Louis, MO) housed in polycarbonate filter holders (Cole Parmer; Chicago, IL) and connected to Luer-Lok™ BD syringes (1 mL, Franklin Lakes, NJ), and suspended on a home-built manifold. Prior to insertion into filter holder, nitrocellulose filters were wetted with filtered water.

For isolation of protein, juice samples (2 mL) were clarified by centrifugation (5 min, 13000g), and 250 µL added to individual 1.5 mL microcentrifuge tubes, followed by 25 µL of a 1M Tris, 100g/L SDS buffer and 100 µL of a 500 g/L TCA solution. Samples were briefly vortexed and allowed to sit for 5 min before loading into syringe filters. The microcentrifuge tubes were rinsed with 300 µL of 60 g/L TCA, and the wash solution used to rinse the residual protein from the tubes added to the syringe prior to filtration.

Following filtration, filter papers with bound protein were transferred to Falcon® twenty-four-well tissue culture plates for the successive staining/destaining baths. Staining utilized 1 mL of 1 g/L Amido Black in 9:2:9 methanol:acetic acid:water for 10 min. After 10 min, filters were transferred into a well of filtered water, followed by a well of destaining solution consisting of

90:2:8 methanol: acetic acid: water to remove excess Amido Black. The destaining protocol was repeated three times, after which filters were transferred to 2 mL microcentrifuge tubes containing 1.5 mL of elution buffer (50% v/v EtOH, 25mM NaOH, 0.05mM EDTA). Filter papers were incubated in the elution buffer for 30 min, with intermittent periods of vortexing. After 30 min, absorbance at 630 nm was measured using an Agilent 8453 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA). Concentrations were calculated based on a standard curve prepared from bovine serum albumin in filtered water (0-35 μg of protein, 5 μg increments, analyzed in triplicate).

Tannin Quantitation in Fruit and Wines

Three frozen 20-berry samples were randomly sampled from every harvested cultivar, weighed, defrosted at 4° C, and dissected into skins, seeds and flesh using forceps and a scalpel. Skin and seeds samples were weighed and tannin was extracted from the skins and seeds using a 70% acetone solution. Samples were agitated on an orbital shaker overnight in the solvent solutions. After the extraction period, 2 mL of the extract were removed and the acetone evaporated under reduced pressure at 30 °C. Samples were reconstituted in filtered water (2 mL), and CT quantified by protein precipitation using the method of Harbertson et al.[30]

For experiment 2, to assess differences in CT across protein removal treatment groups for Maréchal Foch wines where CT fell below the limit of quantitation for the Adams-Harbertson assay, HPLC-phloroglucinolysis was performed as previously described by Manns et al.[34]

Modeling Tannin Adsorption to Protein

The adsorption of CT to proteins was modeled by the linear form of the Freundlich equation,[121] similar to our previous work.[118]

$$log(q_s) = \log K_F + b_F \log [C_S]$$

Where q_s is the mass ratio (mg/mg) of CT adsorbed to the amount of protein in a volume of wine, C_s is the equilibrium concentration of CT left after fining (mg/L), K_F (L/mg) is an empirical constant that represents the adsorption capacity of the fining agent (protein), and b_F is dimensionless and represents the adsorption intensity.

A plot of log (q_s) vs log [C_s] was constructed using JMP Pro 11 (by SAS, Cary, NC), and K_F and b_F determined from the intercept and slope, respectively.

Statistical Analysis

All statistical testing and modeling for this study was performed using JMP Pro 11 and 12 (by SAS, Cary, NC), with a Type 1 error rate set to 0.05. One-way ANOVA and Tukey's post hoc HSD was used to evaluate differences in wine CT, skin CT, seed CT, total berry CT, %CT extracted, and juice protein across grape samples. Student's t-test was used to evaluate differences between groups of samples from CA and NY. For experiment 2, a repeated measures one-tailed t-test was performed to evaluate if a treatment increased tannin as compared to the control, and one-tailed t-tests were also used to evaluate if a treatment increased tannin concentration as compared to the control wine at each time point.[122]

Condensed Tannin Isotherm

An empirical tannin isotherm was generated using the slope and intercept obtained from fitting the Freundlich equation to experimental data. Microsoft Excel (v. 14.6.3) was used to generate iterations of juice protein (mg/L) for given concentrations of grape CT and wine CT.

RESULTS AND DISCUSSION

Experiment 1, predicting tannin extractability from initial grape tannin and juice protein

We have previously demonstrated that retention of exogenous CT added to finished wines is inversely correlated with wine protein concentration, apparently because these proteins bind and

precipitate CT.[118] Because the major CT binding proteins in wine were of grape origin, we hypothesized that grape protein may limit tannin extraction during fermentation, too. Multiple cultivars from both CA and NY were vinified under identical conditions, and concentrations of CT in fruit and wine, protein in juice, and % CT extracted during fermentation for each grape source were determined (Table 4.1), as well as basic juice chemistry (Supplementary Table S4.1, Appendices).

TABLE 4.1 Fruit Condensed Tannin, Wine Condensed Tannin, and Juice Protein for 2014 Harvest Samples

Cultivar	Location	Wine Tannin [1]	Skin Tannin [2]	Seed Tannin [2]	Total Tannin [2]	% Tannin Extracted	Juice Protein [3]
Cabernet franc	CA	241.5 ± 71.3 bcd	0.252 ± 0.046 cd	0.747 ± 0.510 ab	1.000 ± 0.466 abc	14.79 ± 4.4 cd	65.0 ± 6.1 d
Cabernet Sauvignon	CA	286.8 ± 22.0 b	0.749 ± 0.049 a	0.394 ± 0.048 bcd	1.143 ± 0.017ab	14.80 ± 1.1 cd	56.7 ± 7.6 d
Lemberger	CA	68.7 ± 32.3 f	0.219 ± 0.018 cde	0.406 ± 0.167 bcd	0.624 ± 0.171 cdef	7.65 ± 2.2 def	106.3 ± 37.6 bc
DeChaunac	CA	66.7 ± 28.9 f	0.296 ± 0.030 c	0.243 ± 0.067 cd	0.539 ± 0.039 cdef	8.24 ± 2.5 def	67.3 ±4.5 d
Maréchal Foch	CA	50* f	0.069 def	0.376 bcd	0.445 cdef	2.40 ef	199 a
Dornfelder	CA	50 * f	0.130 ± 0.007 def	0.430 ± 0.250 bcd	0.560 ± 0.244 cdef	1.89 ± 0.6 f	123.3 ± 10.9 b
Regent	CA	175.7 ± 16.6 cde	0.092 ± 0.016 ef	0.347 ± 0.084 bcd	0.439 ± 0.094 def	24.03 ± 2.3 b	55.0 ± 4.4 d
Cabernet franc	NY	257.1 ± 22.4 bc	0.525 ± 0.129 b	0.353 ± 0.075 bcd	0.877 ± 0.055 abcd	17.58 ± 1.5 bc	16.4 ± 7.9 ef
Cabernet Sauvignon	NY	334.1 ± 15.5 b	0.774 ± 0.041 a	0.234 ± 0.041 cd	1.001 ± 0.037 abc	19.88 ± 0.9 bc	11.0 ± 6.9 f
Lemberger	NY	140.7 ± 16.5 def	0.223 ± 0.009 cde	1.125 ± 0.218 a	1.348 ± 0.209 a	6.26 ± 0.7 ef	59.0 ± 2.0 d
DeChaunac	NY	50 * f	0.197 ± 0.043 cde	0.144 ± 0.043 cd	0.341 ± 0.060 f	5.21 ± 1.5 ef	52.3 ± 5.0 de
Maréchal Foch	NY	50 * f	0.105 ± 0.012 ef	0.290 ± 0.049 bcd	0.394 ± 0.059 ef	4.06 ± 1.9 ef	113.3 ± 14.3 b
Chancellor	NY	127.3 ± 27.3 ef	0.513 ± 0.078 b	0.118 ± 0.021 cd	0.631 ± 0.068 cdef	12.11 ± 2.6 cde	57.0 ± 8.7 d
Rougeon	NY	50 * f	0.159 ± 0.024 cdef	0.024 ± 0.004 d	0.183 ± 0.021 f	2.84 ± 0.8f	71.7 ± 4.0 cd
Baco noir	NY	50 * f	0.054 ± 0.006 f	0.578 ± 0.147 bc	0.631 ± 0.143 cdef	1.93 ± 0.2 f	72.7 ± 5.0 cd
Vincent	NY	502.0 ± 63.7 a	0.426 ± 0.036 b	0.339 ± 0.032 bcd	0.765 ± 0.063 bcde	39.36 ± 5.0a	22.2 ± 13.2 ef

Data listed in each column are the mean ± standard error, compared by ANOVA and followed by Tukey's honest significant difference test. Different letters within columns denote significant differences ($p<0.05$). [1] mg/L CE, " *" denotes CT measurement below the limit of quantitation for the protein precipitation assay; [2] mg CE/g berries; [3] mg/L

There was no significant effect of location (CA vs NY) on wine CT or total CT in fruit ($p=0.25$ and 0.91, respectively), but "cultivar" had a significant effect on both parameters ($p<0.001$). In agreement with our previous work, Cabernet Sauvignon (from both NY and CA) had the greatest quantities of skin CT by weight, relative to the other varieties in this study.

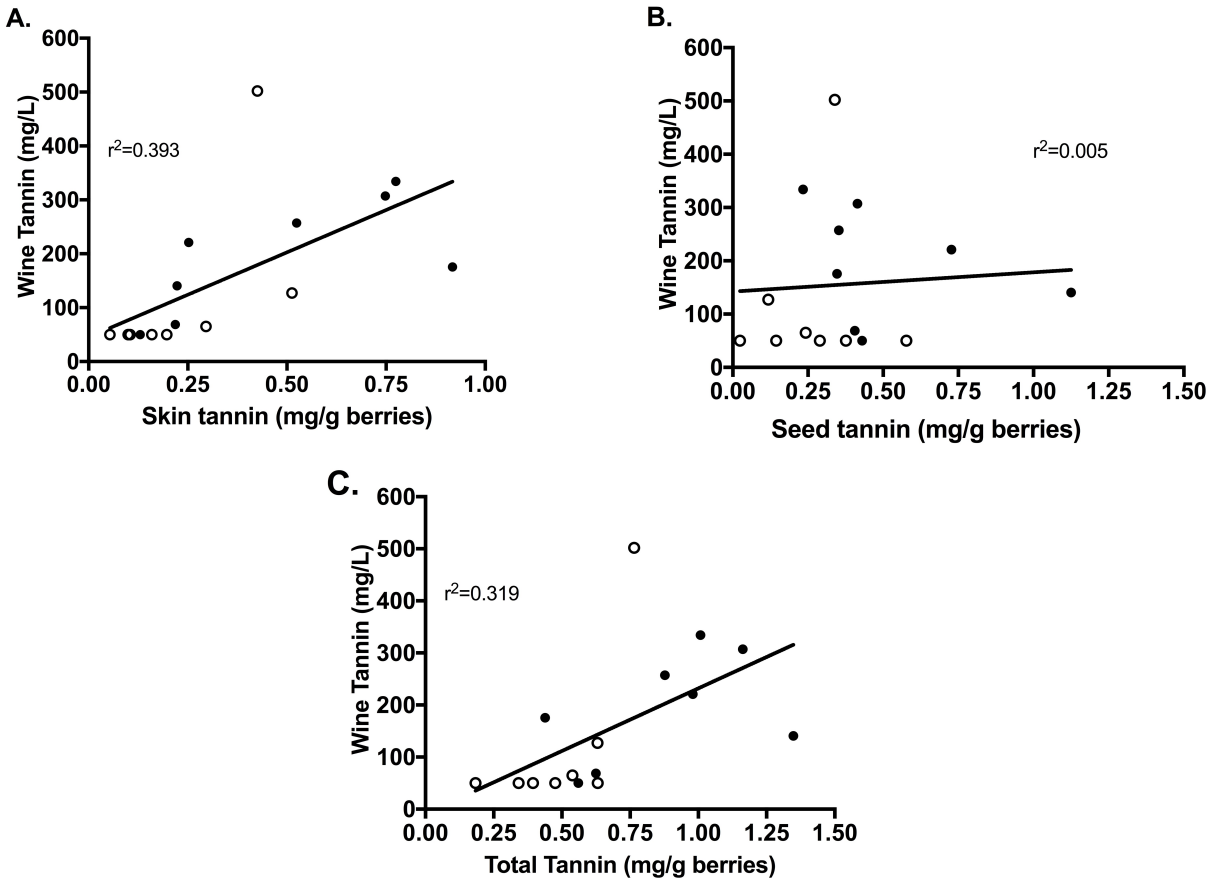
Within grapes from NY, juice from interspecific hybrid varieties had over twice the amount of protein than those from *V. vinifera* (58.8 vs 28.8 mg/L), and had slightly more than half the amount of skin CT and total CT (skin CT 0.268 vs 0.507 mg CT/g of berries, total CT 0.530 vs 1.08 mg CT/g berries). Vincent, an interspecific hybrid grape grown in NY, had significantly higher CT in wine than all other samples from both NY and CA. Although it did not have the highest quantity of CT in skins or seeds, it was in the lowest ranking group for juice protein, corresponding to the highest percent of CT extracted (39.4%). The anomalously low protein in Vincent as compared to other hybrids may be because it has close to 75% *vinifera* parentage, as opposed to 50% or less for the other hybrids under study, and therefore could be expected to have more *vinifera*-like characteristics. Excluding Vincent from the analysis, wines produced from interspecific hybrid grapes also had significantly lower quantities of CT than those produced from *V. vinifera* ($p<0.001$, 46.3 vs 191.6 mg/L CE). For all hybrid varieties (NY and CA), a correlation was observed between juice protein and °Brix ($r=0.73$, $p<0.001$), while this relationship was not significant for *V. vinifera* varieties.

Samples from CA had significantly higher quantities of juice protein compared to those from NY (85.3 vs 49.8 mg/L). There was no evidence of disease presence on the harvested fruit from either group, but the CA samples had a significantly higher average °Brix (22.0 vs 17.7, see Supplementary Table 1) and pH (4.25 vs 3.38), in agreement with previous work showing that protein increases with ripening.[123]

The relationship between CT in fruit and corresponding wine is depicted in Figure 4.1. Weak or non-significant correlations between skin CT and wine CT ($r^2= 0.393$), seed CT and wine CT ($r^2= 0.005$), and total fruit CT and wine CT ($r^2= 0.315$) are in agreement with many other

studies.[59, 93] Correlations were not improved by performing separate regressions for each region (data not shown).

FIGURE 4.1 Correlations of Skin, Seed, and Total Fruit Condensed Tannin with Wine Condensed Tannin

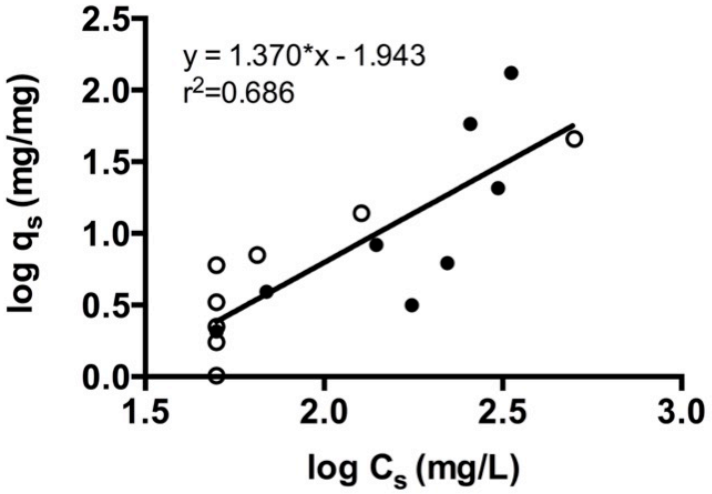


Correlation between wine tannin (mg/L CE) and A. Skin tannin (mg/g CE fresh berries), B. Seed Tannin (mg/g CE fresh berries), and C) Total Tannin (Skin + Seed Tannin, mg/g CE fresh berries)

To assess the impact of juice protein was a limiting factor in CT extraction, we modeled the protein-tannin relationship through the Freundlich equation (Figure 4.2). This model accounts for 68% of the variation in the log ratio of solubilized CT to juice protein (log q_s). Similar to our

previous findings, grape "type" (*V. vinifera* or hybrid) was not a significant factor in the model[118], indicating that the model was valid across species.

FIGURE 4.2 Linearized Freundlich Isotherm-Condensed Tannin Extraction and Condensed Tannin Equilibrium in Wine



Linearized Freundlich adsorption isotherm at 22°C. The log transformed equilibrium wine CT (C_s) is plotted on the x-axis, vs. the log transformed mass ratio (q_s) of CT adsorbed (CT in berries- CT in wines): protein quantity in juice, on the y-axis.

In support of our hypothesis that juice protein limits the extraction of CT into wine, the linear Freundlich equation produced a significant ($p<0.001$), positive relationship between log C_s (equilibrium wine CT), and log q_s (the ratio of solubilized CT to juice protein). Thus, the low tannin extractability observed for certain grapes may be due to precipitation of extracted CT by juice protein during the course of fermentation. Because juice protein is extracted much more rapidly than CT, this problem would not be easily avoided. Additionally, low tannin extractability could also arise from back-binding of CT to insoluble proteins present in grape cell wall material. This hypothesis assumes that soluble juice protein and insoluble cell-wall protein are well correlated, and that juice protein serves as a proxy measurement. Because insoluble protein was not measured, it was not possible to evaluate this hypothesis. However, back-binding

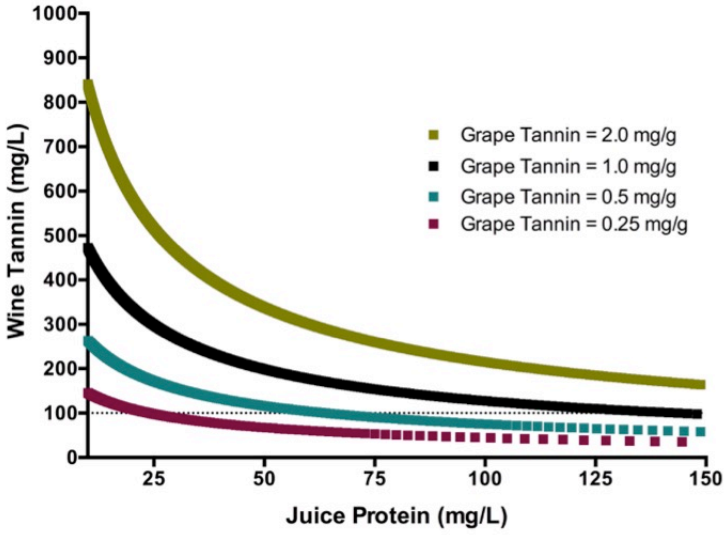
to insoluble cell wall components as a limiting factor in CT extraction has been previously discussed,[40] although grape polysaccharides have been implemented rather than protein.[124]

Protein-tannin interactions can also explain reported variation in CT extraction as a function of solvent choice. In our current work, we employed a 70% acetone extraction on grape skins and seeds to subsequently quantify CT by protein precipitation,[30] as this solvent system is reported to give greater CT extraction (particularly for high mDP fractions) than alcohol based extractions.[125] Similar results were observed when using 50% (v/v) aqueous acetone as a co-solvent in the butanol-HCl-iron assay.[126] Weaker solvent systems, such as the wine-like medium employed by Bindon and colleagues,[42] are reported to extract less CT and provide better correlations between grape and wine CT. The authors proposed that exhaustive solvent extractions would not account for CT back-binding to polysaccharide cell wall constituents during fermentation, but an additional explanation could be that CT extraction is limited by binding to soluble or insoluble grape protein, at least in high-protein grapes. Bindon and coworkers have shown that 70% acetone can fully disrupt non-covalently linked CT from proteinaceous precipitates from red wine,[127] whereas methanol has little effect on protein-CT precipitation.[102] A recent study utilizing methanol to extract CT from grape tissues reported that polymeric CT in grape skins was ~10 fold higher in *V. vinifera* than hybrid grapes,[101] while we observed smaller or non-significant differences between these classes in both our current work and in a previous study.[91] The lower tannin extractability observed for interspecific hybrids during fermentation or in alcoholic media may therefore arise from higher concentrations of proteins in these grapes.

Using the best-fit Freundlich model, CT isotherms were generated for arbitrary levels of grape CT (Figure 4.3). As the steep negative slope of the curve implies, juice protein and CT

appear to participate in cooperative binding, and small increases in juice protein can cause large decreases in tannin extraction. Other studies have suggested positive cooperative binding between CT and protein, in which binding of CT leads to a conformational change in the protein, exposing more binding sites for other CT.[4, 128] The shallow slope of the curve at high protein concentrations (>75 mg/L) may explain the low recovery of pre-fermentation CT additions – particularly for interspecific hybrids.[34]

FIGURE 4.3 Condensed Tannin Isotherm



Tannin isotherms calculated from best-fit Freundlich model from Figure 4.2, plotted as the amount of tannin extracted into wine (y- axis) vs. the amount of protein in juice (x- axis, mg/L) for a given amount of fruit tannin.

A caveat with the reported work is that fermentations used frozen and thawed fruit for practical reasons involving sourcing fruit from multiple sites. However, freezing is known to increase the rate of CT extraction from skins,[129] and a similar effect could be hypothesized for proteins. Additionally, as shown in Experiment 2, freezing can result in protein precipitation. This concern does not change our conclusion that grape protein-CT interactions can limit final wine CT, since the phenomenon can be well modeled by the Freundlich equation, and because we had previously observed that wine protein could be predicted from grape CT and juice

protein using a similar model.[118] However, in conventional winemaking using fresh grapes, the kinetics of CT extraction would likely be slower and the juice protein content could be either higher or lower. The consequences of these differences on final wine tannin are unknown.

Experiment 2, pre-fermentation protein removal and tannin extractability

Results from Experiment 1 suggested that pre-fermentation protein removal treatments should increase CT extraction. This hypothesis was investigated on two cultivars – a *V. vinifera* (Lemberger) and an interspecific hybrid (Maréchal Foch). In agreement with Experiment 1 of this study, CT in Maréchal Foch fruit (skin CT= 0.23 mg/g, seed tannin= 0.94 mg/g) was lower than Lemberger (skin CT= 0.38 mg/g, seed CT= 1.23 mg/g). In addition, Maréchal Foch juice had higher a protein concentration than Lemberger (avg=150.7 mg/L vs 93.7 mg/L, $p<0.05$), in agreement with previous work.[118] Skin and seed CT values from Experiment 2 were also generally comparable to Experiment 1 data (Table 1) and to previous reports,[58] with the exception of seed CT from Maréchal Foch in the 2014 vintage (0.29 mg seed CT/g berry). This was also considerably lower than both the seed CT from Maréchal Foch in the 2015 vintage (Experiment 2, 0.94 mg CT/ g berry), as well as earlier work by our group on Maréchal Foch (0.58-0.83 mg CT/g berry).[49] The reason for this lower value is unknown. However, seed number was not determined, and previous work by Harbertson and colleagues has shown that seed number is major factor responsible for differences in seed CT across fruit.[30]

Heating, flash freezing, tannin addition and bentonite treatments all significantly reduced the juice protein concentration of both varieties under investigation (Table 4.2, $p<0.05$). Bentonite, which is widely used in the wine industry for removal of grape-derived haze proteins,[121] had the greatest effect in both cultivars (85% and 78% decrease in Maréchal Foch and Lemberger respectively), comparable to results by Sauvage et al.[130] According to this previous study,

thaumatin-like proteins were most likely to persist after bentonite treatment, representing a pool of protein that has been previously shown to bind commercial CT added to wines.[118] Both heating and flash freezing were expected to denature and remove proteins, and both resulted in modest decreases in protein via precipitation. Heat treatment removed 61% of Lemberger juice protein and 67% of Maréchal Foch juice protein, while freezing removed 29% and 55%, respectively. Comparable decreases were also observed for the commercial CT addition (56% protein reduction in Lemberger, 60% reduction in Maréchal Foch.)

TABLE 4.2 Protein Concentration Before and After Juice Treatments, and in Wine at Bottling

Maréchal Foch	**Before Treatment**	**After Treatment**	**Bottling**
Heating	175.8±15.2 X	57.6 ±6.5 Y b	71.2±5.7 Y b
Freezing	153.8±12.3 X	68.7±2.9 Y b	61.1±3.9 Y bc
Tannin addition	139.6±8.7 X	55.6±8.5 Y b	48.7±2.2 Y bc
Bentonite	138.6±7.4 X	20.9±2.8 Y c	37.8±3.8 Y c
Control	145.7±13.8	141.4±6.8 a	138.6±8.0 a
Lemberger			
Heating	84.9±9.5 X	33.3±6.6 Y bc	15.9±2.3 Y b
Freezing	86.6±7.7 X	61.9±8.9 Y b	20.2±1.8 Z ab
Tannin addition	98.7±11.1X	43.7±5.2 Y bc	29.3±3.7 Y ab
Bentonite	98.2±12.7 X	24.6±4.6 Y c	32.6±4.3 Y a
Control	100.2±5.4 X	99.5±6.8 X a	34.6±3.1Y a

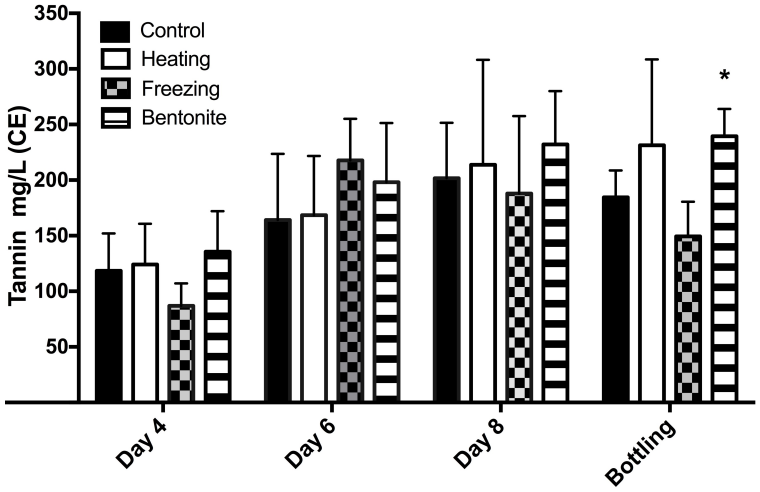
Protein quantitated in mg/L before and after experimental or control treatment, and at bottling. Data given as the mean ± standard error, compared by ANOVA and followed by Tukey's honest significant difference test. Different capital letters within rows and lower case letters within columns denote significant differences ($p<0.05$).

The starting CT concentration in juice from both cultivars was below the limit of quantification for the protein precipitation assay (<100mg/L). The amount of CT extracted from Lemberger during fermentations at 2-day intervals for each treatment group is depicted in Figure 4.4, with a full time course reported in Supplementary Table S4.2 (Appendices). CT quantities remained below the limit of quantification until approximately Day 4 for all the protein removal treatment groups and generally increased until just before bottling, on Day 9. The bentonite

treatment led to significantly higher CT in must across the fermentation ($p<0.05$, repeat comparisons one tailed t-test) and higher CT in wine at bottling on Day 9 ($p<0.05$, one tailed t-test) compared to the control, heating and flash freezing treatments. The tannin addition treatment was not included in these analyses due to the confounding effect of residual tannin, as described below. The observation that pre-fermentation treatment of juice with bentonite results in the largest improvement in tannin extraction is in agreement with the observation that it causes the greatest decrease in juice protein pre-fermentation (Table 4.2). However, this effect was somewhat smaller than expected. The observed increase in CT extraction between bentonite treatment and control was 30%, as compared to the 82% increase expected when using values of 39 mg/L juice protein and 1.61 mg/g CT in the Freundlich model. This muted effect is likely because the pre-fermentation treatment would have removed protein only from the juice, and not from the skins and seeds (or remaining juice), leaving a pool of remaining protein to enter the juice matrix upon remixing. Using a juice yield of 50%, and assuming protein was at comparable concentrations between juice and pomace, the actual protein concentration following recombination of juice/skin/seeds would have been closer to 69.5 mg/L. This would predict a 26% increase in wine CT in the bentonite treatment, comparable to the observed change. In support of this hypothesis, no decrease was observed in protein content at bottling as compared to pre-fermentation (Table 4.2), even though a portion of protein was presumably lost to CT binding. In our previous work investigating CT binding to alcohol-insoluble cell wall fractions from grape skins and flesh, we observed a significant correlation between CT binding and crude protein content in grape flesh cell walls.[91] The solubilization and/or release of cell wall proteins could potentially occur throughout fermentation, leading to additional CT losses via precipitation. Because cell wall fractions were prepared as an alcohol-insoluble residue of grape

flesh in our earlier work, it is unclear how much was actually insoluble (e.g. a structural protein), as opposed to physically entrapped in the cell wall matrix, or soluble but precipitated by the alcohol addition step. PR proteins of plants are typically found in the apoplastic space, cell walls and vacuoles of cells,[131] and would be a soluble pool of protein that could remain entrapped of cell structures to be released throughout the course of fermentation as these cellular structures are broken down. Future work on the distribution (solubilized vs. soluble-but-trapped vs. insoluble) of grape-derived proteins during fermentation should be helpful in rationalizing effects of bentonite or other treatments.

FIGURE 4.4 Lemberger CT Extraction During Fermentation



Wine tannin (mg/L CE) as a function of time for control and protein-removal treatments (heating, freezing, and bentonite) application. Bottling occurred at Day 9. Values represent average of three fermentation replicates, and error bars show standard deviations. A "*" symbol above the bar denotes a significant difference from the control by one tailed t-test ($p<0.05$).

The thermal treatment resulted in no significant effect on CT extraction, and the freezing treatment resulted in significantly lower CT extraction (Figure 4.4), despite the effectiveness of both treatments in removing juice proteins (Table 4.2). Both heating and freezing will result in protein denaturation,[132, 133,134] and any residual denatured proteins may be more efficient at

binding CT.[130] Additionally, protein extracted from skins and seeds during fermentation may have limited CT extraction, as discussed for the bentonite treatment.

TABLE 4.3 CT in Lemberger Wines

Treatment	At Bottling	After 6 mo Storage
Heating	231.4±44.5	167.9±46.6
Freezing	149.4±17.9	100.5±18.9
Tannin Addition	282.3±49.2	211.4±39.1
Bentonite	239.5±14.1 *	181.5±43.2
Control	184.4±14.1	145.9±29.1

Condensed Tannin (mg/L CE) in Lemberger wines as a function of treatment at time of bottling and after 6 months storage. Data are given as the mean ± standard error, * denotes a significant difference from the control wine as determined by a one-tailed t-test.

We utilized BioTan in previous experiments for its relatively high purity (47%)[91] compared to other commercial products.[33] Assuming the level of purity was comparable in our own experiments, a 600 mg/L addition of BioTan to treated juices equates to 282 mg/L CE. The tannin addition treatment to Lemberger juice both removed juice proteins and resulted in higher wine CT than the control, and all of the treatment groups (Table 4.3). The tannin addition group measured 236 mg/L CT following treatment, while other treatment groups and the control did not surpass the limit of quantitation for the Adams-Harbertson assay at this time point. CT subsequently decreased to 103 mg/L the following day and gradually increased as endogenous CT was extracted during fermentation. At bottling, the tannin addition treatment had 282 mg/L CT, significantly higher than the control wines (184 mg/L) and comparable to the bentonite treatment. However, the amount of exogenous CT lost during fermentation (~63%) is comparable with previous reports, which show 50-80% losses in prefermentation CT additions.[34, 35] Thus, in our work, bentonite treatment results in more efficient CT extraction than tannin additions. However, CT decreased during storage across all treatments during storage, and differences in CT was no longer significant after 6 months bottle age (Table 4.3). Similar losses

(~50%) in wine CT during the initial stages of storage have been reported by other authors, and could arise from condensation of CT with anthocyanins or other reactions.[87]

Except for a transient spike in CT for the tannin addition treatment, all of the Maréchal Foch treatments had CT below detection limits throughout the fermentation (data not shown). To assess differences resulting from treatments, an HPLC-phloroglucinolysis method was employed to measure the low levels of CT in Maréchal Foch wines (Supplementary Table S4.3, Appendices), but no significant differences were found between groups after 6 months of bottle storage. This low CT in Maréchal Foch wine likely arises from the compounding effects of low fruit CT and high juice protein in this cultivar. Using the Freundlich model and assuming 50% juice yield for the treated portion of Maréchal Foch juice, we expected the bentonite treatment to yield 165 mg/L CT in wine, vs. 112 mg/L CT in the control. Interestingly, both of these values should have been above detection limits for the Adams-Harbertson assay (100 mg/L). The poorer-than-expected extraction across Maréchal Foch treatments could arise from a number of causes, including the presence of other CT binding grape components, and/or greater CT-binding ability by protein from this cultivar. Our current work considered total protein, and did not separate and quantify individual proteins. In previous work, we quantified individual PR proteins (chitinases, thaumatin-like proteins, etc.),[118] but observed no improvement in modeling retention of exogenously added CT based on individual protein measurements as compared to total protein. Because of the cost and time associated with measuring individual proteins rather than total protein, we chose to measure the latter in this work.

Additionally, the Freundlich model in Figure 4.3 is based on juice protein, which we assumed to be well correlated with total must protein (insoluble and soluble). However, our current work did not determine if soluble or insoluble protein was more important to limiting

wine tannin. We have previously observed that the insoluble grape cell wall material of interspecific hybrids had both higher N content and stronger tannin binding capacity than that that of *vinifera.*[91] Potentially, hybrids like Maréchal Foch could have a high ratio of insoluble to soluble protein, which would result in an overestimation of final wine tannin when using a model based only on juice protein.

In the tannin addition treatment of Maréchal Foch, a 600 mg/L BioTan addition resulted in an increase in CT from undetectable to 145 mg/L. Assuming no background CT, this represents an immediate 49% loss of CT following addition to Maréchal Foch juice, as compared to the 16% loss observed for addition to Lemberger. We observed similarly lower retention of exogenous CT in Maréchal Foch wine as compared to *vinifera* in previous work, which we credited to the higher protein content of Maréchal Foch.[118] The higher concentrations of proteins in wild *Vitis* species (e.g. *Vitis riparia*) or their interspecific hybrids (e.g. Maréchal Foch) as compared to *vinifera* may be related to their better resistance to both abiotic and biotic stresses, carrying potentially important implications for the future grape breeding initiatives to improve wine quality.[106] In addition to differences among cultivars, PR proteins in fruit (and musts) are also reported to increase with greater disease pressure, under humid conditions and after temperature stresses.[94] Interestingly, some of these conditions are also associated with changes in CT, e.g. higher soil water availability is reported to decrease skin CT in grape berries,[135] and this high water condition would be expected in regions with higher humidity and more disease pressure.

In summary, we show that CT extraction during fermentation is well modeled by initial concentrations of both grape tannin and juice protein across multiple regions and cultivars. These suggest that grape-derived proteins are not only important as a contributor to haze in white wines, but that they also likely limit CT extractability in red wine fermentations, and that pre-

fermentation removal of protein can be used to achieve wines with higher CT concentration. Specifically, we show that pre-fermentation bentonite fining can both reduce protein and increase wine tannin concentration. Our approach to protein removal, in which juice was removed from the solids and treated with bentonite prior to recombination with solids and fermentation, may be cumbersome in a commercial winemaking setting. The use of proteases, as has been described for white wine haze prevention[94], could allow for treatment with racking and returning juice, and may also be a useful option for increasing tannin extraction.

CHAPTER 5

FUTURE WORK

Developing a High Throughout Protein Quantitation Method for Juices and Wines

As shown in previous chapters, grape derived proteins in the range of 12-65 kDa impact red wine quality by preventing the extraction and retention of CT.[91, 118] This is particularly evident in the case of red interspecific hybrid wine grapes, which tend to have larger protein quantities and less CT than *V. vinifera*. From the inclusion of native *Vitis* spp. in this work, it is clear that species favored by grape breeders for breeding, such as *V. riparia*, contain the highest amount of soluble proteins.[118] While it is unknown whether the enhanced levels of PR proteins in interspecific hybrids are related to their resistance to disease and cold temperatures, it is evident that they negatively affect wine quality. This dilemma has been previously discussed from the perspective of white wine haze, where others have noted that breeding for disease resistance may also result in breeding for protein instability in white wines.[106]

Rapid tools for quantifying protein in red grape juice, must and wines would thus be valuable for grape breeders and winemakers. In Chapter 3, protein was precipitated from juices and wines using ammonium sulfate, de-salted by dialysis, separated by SDS-PAGE, and quantified by densitometry. Although the SDS-PAGE method is a reliable standard for protein quantitation,[136] the method requires one week and would not be practical for routine analysis of large sample populations, or to make time-dependent fermentations decision during harvest (primary fermentation can be completed in that time!). The qualitative information provided by the SDS-PAGE regarding the types of proteins is also too detailed, as my previous work showed that total quantity of protein best predicts CT extraction (Chapter 4) and retention (Chapter 3).

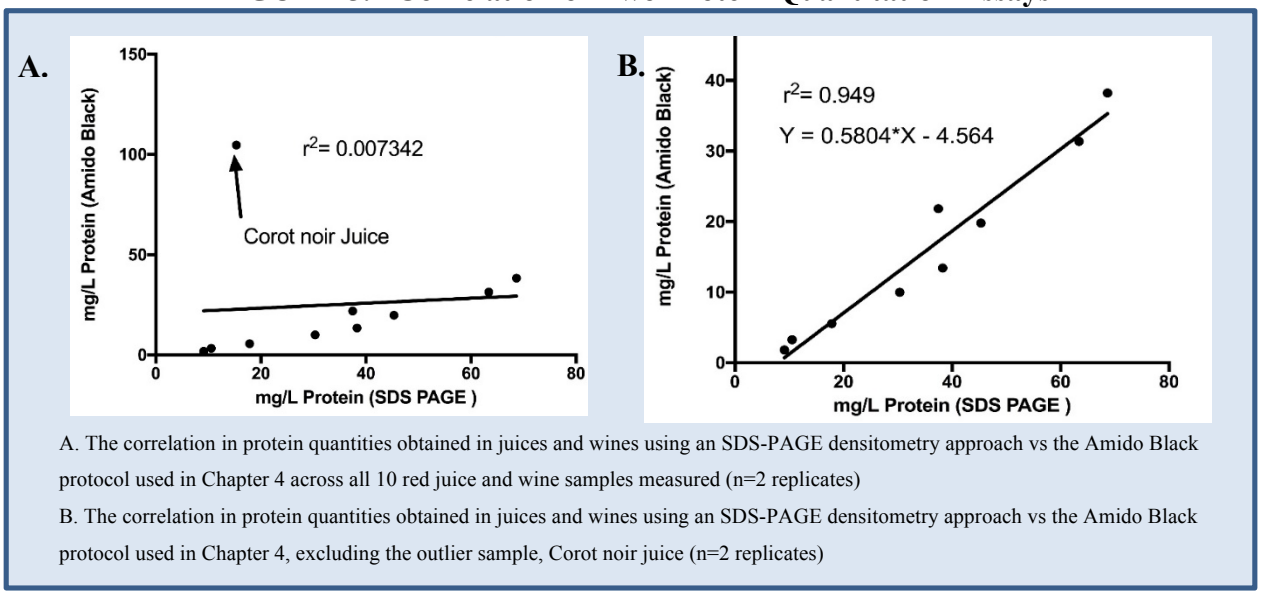
Quantifying proteins in juices and wines is particularly difficult using standard methods such as Bradford, Lowry, and BCA due to the presence of interferences from phenolics, ethanol and polysaccharides.[137] This is especially evident when protein concentrations in juice or wines are low (below 10 mg/L).[136] Additionally, there is no existing grape derived protein standard to calibrate assays and account for sequence dependent biases in staining between the sample and calibration protein or differences in glycosylation. BSA has been a ubiquitous protein used for assay standards. However, it differs significantly from grape derived proteins in that, sequentially it is not derived from plants, and it is also not glycosylated like most PR proteins.

In Chapter 4, protein in juice was quantified using a modified Amido Black protocol that precipitates protein and subsequently adsorbs the protein to a nitrocellulose membrane.[120] This allows the majority of interfering substances to pass through the filter. The nitrocellulose membrane is then treated with Amido Black stain, which binds the adsorbed protein. The previous report investigating this method described little to no interferences from pectin, glutathione, or common wine phenolic species.[120] The original described method by Weiss and Bisson involved dripping samples on a vacuum filter, but the low throughput of the method meant it was poorly suited for the number of samples to be processed for the study described in Chapter 4. Therefore, the assay was modified from using a vacuum to forcing the sample through a nitrocellulose membrane using a filter holder and syringe. This allowed for multiple (16) samples to be pushed through at once, after the construction of a wooden manifold to hold the syringes. Details regarding the assay can be found in Chapter 4.

The correlation between the Amido Black protocol and SDS-PAGE densitometry-based approach for protein quantification was improved by excluding one outlier sample (Figure 5).

The samples in this dataset were red juices and wines from four cultivars under investigation for an industry sponsored project.

FIGURE 5.1 Correlation of Two Protein Quantitation Assays



A. The correlation in protein quantities obtained in juices and wines using an SDS-PAGE densitometry approach vs the Amido Black protocol used in Chapter 4 across all 10 red juice and wine samples measured (n=2 replicates)

B. The correlation in protein quantities obtained in juices and wines using an SDS-PAGE densitometry approach vs the Amido Black protocol used in Chapter 4, excluding the outlier sample, Corot noir juice (n=2 replicates)

The outlying sample depicted in Figure 5.1A had a profound effect on the correlation between methods, but may be explained in light of previous results and discussions in this work. In Chapter 2, cell wall material was prepared as an ethanol insoluble fraction from grape flesh and skin.[91] Noting the good correlation between crude protein and CT binding in grape flesh (r^2= 0.597, Figure 2.3), it was also apparent that the Corot noir flesh samples, specifically, had statistically higher quantities of protein compared to all of the other varieties under investigation (ave 13.9% by weight. Table 2.2). As it was mentioned in Chapter 3, only proteins that enter the SDS-PAGE gel can be stained and quantified by densitometry, and this excludes some heavily glycosylated proteins (*proteoglycans*). Similar to yeast-derived mannoproteins, it is possible that protein-rich juice soluble cell-wall fragments (e.g. arabinogalactans) with significant carbohydrate moieties were unable to migrate on the SDS-PAGE gel, yet were able to bind to the

nitrocellulose filters for quantitation via the Amido Black protocol. This may be considered a variety specific effect for Corot noir juice as the correlation between the two methods for all of the other nine samples, including Corot noir wines, was r^2=0.949 (Figure 5.2B). It is likely that the protein rich cell wall material strongly bound with CT and was fined out during vinification, therefore not appearing in wine. In support of this, in Chapter 2, alcohol-insoluble cell wall material from Corot noir was the strongest binder of CT on a by-weight basis. Alternatively, the effect of ethanol may account for the absence of this effect in Corot noir wine samples.

Even after manifold construction and method modification, the sample throughput of the Amido Black assay to quantify protein in replicates was not ideal to screen large mapping populations (150+ individuals). It was projected that if the format of the assay could be modified to a 96-well plate, automation could be applied to further enhance the sample throughput and screening capabilities. The first step in this development would be to secure 96-well nitrocellulose filter plates, however, these were not readily commercially available. Therefore, other protein binding chemistries were investigated for the development of a 96-well plate based method.

PVDF, or polyvinylidene difluoride, is a common membrane used for Western Blotting and binds proteins via hydrophobic interactions. It is less brittle than nitrocellulose, has a higher protein binding capacity, is compatible with a greater range of solvents, and is readily available in a 96-well plate format from a variety of distributors. One of the major differences between PVDF and nitrocellulose membranes is the need to pre-wet PVDF with an alcohol prior to use with aqueous media.

The experimental protocol was adapted from the published Weiss and Bisson Amido Black method to quantify protein using 96-well PVDF membrane plates and can be found below.

PVDF 96-Well Plate Assay to Quantitate Protein in Juices and Wines

Materials:

96-well vacuum manifold

Plate reader

Bucket centrifuge

Staining Tray

PVDF 96-well filter plate (Multiscreen® HTS, 0.45µm Hydrophobic High Protein Binding Immobilon-P Membrane, Ref: MSIPS4510)

96-well culture plates

Multichannel pipette (optional)

Solutions:

70% (v/v) Ethanol

1M Tris, 100 g/L SDS

500 g/L TCA (trichloroacetic acid)

60 g/L TCA

1 g/L Amido Black in 9:2:9 Methanol:acetic acid:water (staining solution)

90:2:8 Methanol: acetic acid: water (destaining solution)

25mM NaOH, 500mL/L EtOH, 0.05mM EDTA (elution solution)

Procedure:

1. Prepare the PVDF membrane in the filter plate:

Add 75 µL of 70% ethanol to wells on the PVDF filter plate. Allow liquid to sit for at least 1 minute to wet the membrane. Apply a weak vacuum to draw ethanol solution through. While membranes are wetted, rinse wells twice with filtered water, drawing liquid through the plate with a weak vacuum. Do not dry out the membrane, the wells should appear translucent before adding samples.

2. Prepare a standard curve:

Make a 1 µg/µL BSA solution in filtered water or buffer. Use this stock solution to make a range of BSA standards, from 0-25 µg BSA in 200µL volumes, in triplicate to the 96-well culture plate.

3. Condition samples and BSA curve

Add 200 µL of sample juice or wine to sample wells in the 96-well culture plate, in triplicate. To both samples and standard curve tubes, add 20 µL of the Tris/SDS solution, followed by 80 µL of the 500 g/L TCA solution. Mix using pipette or gentle swirling[1].

3. Bind proteins in samples and standards to filtration plate:

a. Add the entire volume (300 µL) to sample wells on the filter plate and apply a gentle vacuum to pull liquid through.

b. Once the sample is pulled through the filter, rinse the sample wells with 200 µL of 60 g/L TCA and load onto corresponding sample well on the filter plate. Pull through filter plate with a gentle vacuum.

4. Stain: Add 50 µL of amido black staining solution to each well on the filter plate and let stand for 10 min.

5. Wash Away Excess Stain:

Carefully dump excess stain from plate and briefly rinse the plate with filtered water. Immerse the filter plate in a dish (staining tray) of the destaining solution, making sure the solution fills all the wells. Allow the plate to destain for a total of 10 min., refresh with new destain solution three times (approximately every three min.) during this time.

The "zero protein" standard filter wells should appear white by the end of 10 mins, with increasing blue color with added protein across your standard curve samples. Remove plate from destaining solution, carefully dump off excess destaining solution and blot the bottom of the plate dry with a clean paper towel.

6. Elute Bound Stain:

Set the filter plate on top of a clean 96-well cell culture plate, aligning the wells. Add 150 µL of elution solution to each well and let stand for at least 10 min. Use bucket centrifuge to pull the elution buffer through the filter and into the underlying wells of the culture plate. Repeat with another 150 µL of elution solution, making sure the solution gets completely drawn through the filter and ends up in the culture plate well directly underneath it. There should not be any blue color left on the filter plate after this step (although you may see some yellow/brown from phenolics).

7. Measure

Use plate reader to measure absorbance in the culture plate wells at 630 nm. Using the standard curve on the plate, calculate the amount of protein in samples.

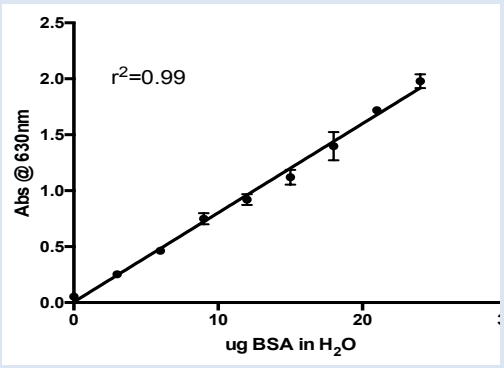
[1] Alternatively, it is possible to use less sample if the same ratios of other added solutions used, e.g. 100 µL sample with 10 µL Tris/SDS and 40 µL of the 500 g/L TCA solution

[2] Sometimes excessive polysaccharide or protein can overload the filter and filtration may slow or stop. Either diluting the sample, or filtering it through a low protein binding filter (PES, polyethersulfone), should resolve the issue.

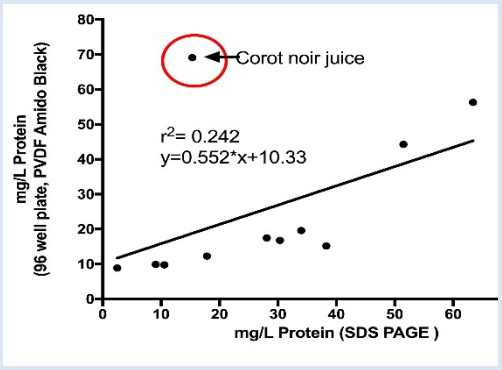
FIGURE 5.2 Protein Quantitation Using a 96-Well PVDF Filter Plate

A.

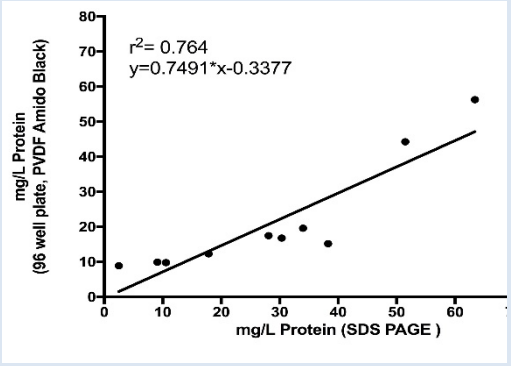
B.



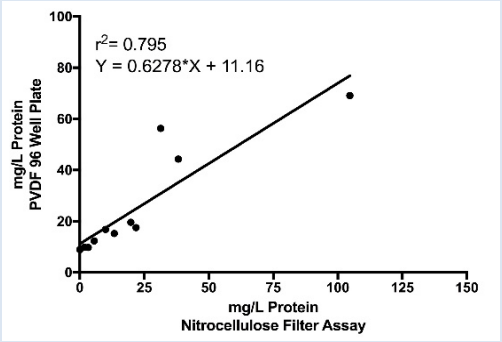
C.



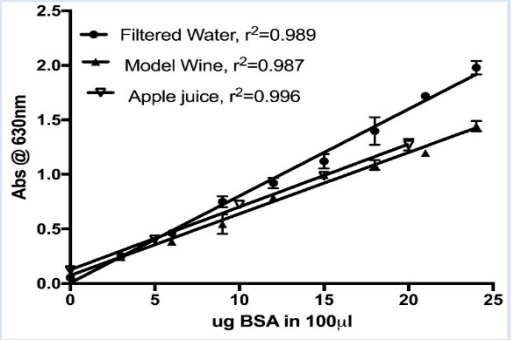
D.



E.



F.



A. A BSA standard curve, in triplicate, after eluting the Amido Black dye into the 96-well culture plate for quantitation
B. Absorbance vs BSA quantity for a standard curve, in triplicate.
C. The correlation in protein quantitation in juices and wines obtained using a standard SDS PAGE densitometry approach vs the PVDF 96-well plate Amido Black protocol
D. The correlation in protein quantitation in juices and wines obtained using a standard SDS PAGE densitometry approach vs the PVDF 96-well plate Amido Black protocol, excluding the outlier sample, Corot noir juice
E. The correlation between the modified nitrocellulose filter assay described in Chapter 4 and PVDF 96-well plate assay
F. The matrix effects on the PVDF 96-well plate assay, as illustrated by BSA added to either filtered water, model wine, or apple juice

Results of preliminary experiments with the PVDF 96-well plate method in comparison to a standard method (SDS-PAGE and densitometry) of protein quantitation, and the adapted Amido Black protocol using nitrocellulose filters (as described in Chapter 4) are depicted in Figure 5.2. From preliminary studies, it is evident that increasing quantities of a BSA standard in filtered water yields excellent linear results for quantitation (Figure 5.2A and 5.2B). For the new assay, each PVDF plate of samples incorporated a standard BSA curve for quantitation to account for differences between plates (similar concept to ELISA assays). The results of quantitation using the PVDF plate assay for the 10 red juice and wines samples are compared to the standard SDS-PAGE assay in Figure 5.2C and 5.2D. Similar to the results from the nitrocellulose filter assay (Figure 5.1A and 5.1B), the Corot noir juice sample gave a higher number for total protein in the PVDF plate assay than the SDS-PAGE method (69.1 vs 15.4 mg/L). This is the same outlier discussed in Figure 5.1A, and may be the result of protein rich soluble cell wall materials that are unable to migrate on SDS-PAGE. Figure 5.2E depicts the correlation, r^2=0.795, between the nitrocellulose filter assay used in Chapter 4 and the PVDF 96-well plate assay. Taken together with Figure 5.2F, it is possible that matrix affects, or perhaps differences in the nature of protein, can slightly influence quantitation on the PVDF membrane by limiting the binding interaction to the filter. Given the nature of the interaction between the proteins and PVDF is hydrophobic, the sample matrix, even after dilution with the assay buffers, could potentially provide interferences. This is supported by the apparent differences in slope between BSA in filtered water and model wine (12% ethanol), both run on the same PVDF filter plate, in Figure 5.2F. Speculatively, an alternate explanation is that the protein binds in a different conformation to each membrane altering the amount of dye accessible binding sites for quantitation.

This preliminary development demonstrates that protein quantitation via a 96-well plate is feasible, opening up the possibility for higher throughput. However, further work is needed to investigate the effects of sample matrix and validate against a standard method of quantitation. A commercially available 96-well nitrocellulose filter plate would also be useful.

Other Future Work

1. In Chapter 2, crude protein in cell wall materials derived from grape flesh was well correlated with CT binding. In the next chapter, PR proteins were revealed as a soluble component responsible for CT fining. Approximately 60% of the variation in CT extraction and retention was attributed to total soluble proteins, but the contribution of protein in grape solids is unknown. Therefore, future work to assess the importance of structural cell wall proteins in CT extraction would be valuable to make a more complete model of CT extraction and retention.

2. In Chapter 4, bentonite treatment was demonstrated to remove the most protein from Lemberger juice, and consequently lead to the best improvement in CT extraction. Currently, the industry standard to remove proteins from wine is bentonite, an absorbent clay formed by the breakdown of volcanic ash. Although effective, bentonite can negatively affect wine quality by stripping aroma compounds, while up to 10% of the treated volume is lost in the settled lees[138]. In addition to concerns about quality and lost wine volume, the labor input and associated health, safety and environmental risks also pose expenses. As of the year 2000, it was estimated that bentonite fining cost the global wine industry $300-500 million per year. Therefore, other treatments that remove grape-derived proteins from juices and wines, preserve wine quality, and are ecologically and economically friendly, could prove beneficial. Ideally, a regenerable and

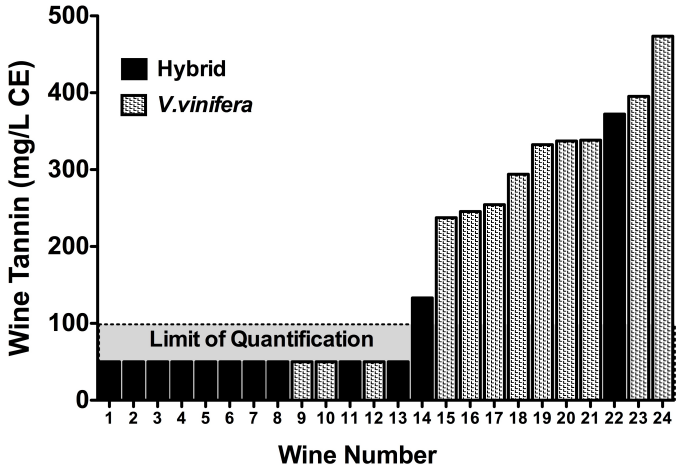
selective protein binding column or fining agent would allow for a reusable, eco-and wine-friendly solution.

3. Finally, once a high-throughout protein quantitation method is properly validated, viticultural research and breeding efforts to characterize the environmental and genetic underpinnings of protein expression in grape fruit will uncover valuable knowledge that can ultimately be used to improve the quality of red wines. In conjunction with ongoing research surrounding grapevine phenolics, enhanced knowledge surrounding protein status can allow CT management to begin with the proper selection of *Vitis* spp for planting, continue with an appropriate viticultural management program, and facilitate fine-tuning in the cellar to achieve quality goals.

APPENDICES

CHAPTER 2 SUPPLEMENTARY MATERIALS

Figure S2.1 CT in 24 Red Hybrid-based or *V.vinfera* Wines



Protein precipitable tannin in 24 wines (12 hybrid-based, 12 vinifera), from the Finger Lakes AVA region of NY. Twenty of the wines were commercial and the remainder were produced at Cornell University (Geneva, NY) as research wines

TABLE S2.1 Tannin in individual grape and wine samples

Classification	Cultivar	Wine Tannin (mg/L CE)	Skin Tannin (mg/g berries CE)	Seed Tannin (mg/g berries CE)	Total Tannin (mg/g berries)	Extractability (%)
French American Hybrid	Baco noir	49.0 ± 15 c d	0.178 ± 0.013 d	0.454 ± 0.029 c d e	0.632 ± 0.025 d e	5.69 ± 1.8 c d
	Leon Millot	ND d	0.221 ± 0.006 c d	0.594 ± 0.011 b c d	0.815 ± 0.0095 c d e	2.25 ± 0.0026 d
	Marechal Foch	83 ± 8 c d	0.248 ± 0.046 c d	0.763 ± 0.033 b c d e	0.969 ± 0.13 c d e	6.26 ± 1.1 c d
	DeChaunac	ND d	0.178 ± 0.013 d	0.264 ± 0.033 de	0.441 ± 0.037 e	4.15 ± 0.35 c d
	Average	**45.4 ± 7.2 B**	**0.206 ± 0.014 B**	**0.519 ± 0.211B**	**0.714 ± 0.229 B**	**4.58 ± 0.90 B**
Neo-American Hybrid	Noiret	354 ± 39 a	0.785 ± 0.071 a	0.208 ± 0.011 e	0.993 ± 0.070 b c d	26.1 ± 3.4 a
	Corot noir	113 ± 55 b c d	0.340 ± 0.074 b c d	0.917 ± 0.15 a b	1.257 ± 0.098 b c	6.63 ± 3.3 c d
	Average	**233.7 ± 55 A**	**0.562 ± 0.096 A**	**0.563 ± 0.355 A B**	**1.13 ± 0.189 A**	**16.4 ± 6.9 A**
V.vinifera	Pinot noir	358 ± 33 a	0.559 ± 0.12 a b	1.190 ± 0.064 a	1.749 ± 0.11 a	15.0 ± 1.7 a b c
	Merlot	259 ± 46 a b	0.774 ± 0.047 a	0.447 ± 0.057	1.221 ± 0.10 b c	15.6 ± 3.1a b c
	Lemberger	158 ± 28 b c d	0.185 ± 0.030 d	1.22 ± 0.053 a	1.409 ± 0.061 a b	8.24 ± 1.5 c d
	Sangiovese	174 ± 19 b c	0.517 ± 0.047a b c	0.440 ± 0.072	0.957 ± 0.10 b c d	13.4 ± 2.0 b c d
	Cabernet Sauvignc	357 ± 30 a	0.593 ± 0.080 a b	0.596 ± 0.051b c d	1.19 ± 0.13 b c	22.0 ± 3.0 a b
	Cabernet franc	183 ± 23 b c	0.355 ± 0.064 b c d	0.763 ± 0.14 b c	1.12 ± 0.025 b c	12.0 ± 2.5 b c d
	Average	**248.2 ± 21 A**	**0.497 ± 0.047 A**	**0.776 ± 0.354 A**	**1.27 ± 0.275 A**	**14.4 ± 2.3 A**

Cultivars or classifications not connected by the same letter denote significant differences ($p<0.05$).

TABLE S2.2 Basic Compositional Data for 2012 Grape Samples

Cultivar	Location	Berry Weight (g)	Soluble Solids (°Brix)	Titratable Acidity (g/L)	pH
Baco noir	Knapp	1.05	22.3	14.80	3.10
	Lakewood	1.18	19.8	16.80	2.97
Leon Millot	Lakewood	1.04	22.2	9.35	3.15
	Fulkerson	1.02	21.8	8.82	3.20
Marechal Foch	Fulkerson	1.08	27.2	9.40	3.50
	Prejean	0.84	26.3	8.43	3.60
DeChaunac	Fulkerson	1.62	20.1	9.93	3.00
	Knapp	1.41	20.1	8.83	3.11
Noiret	Fulkerson	1.83	18.7	7.73	3.17
	Cornell	1.86	19.8	8.68	3.09
Corot noir	Fulkerson	1.41	21.7	6.82	3.55
	Cornell	1.47	19.9	4.25	3.26
Pinot noir	Lakewood	1.22	18.9	7.44	3.10
	Fulkerson	1.36	20.6	10.40	3.03
Merlot	Sawmill	1.68	24.5	6.78	3.51
	Fulkerson	1.33	21.9	6.65	3.35
Lemberger	Knapp	2.03	20.5	6.61	3.16
	Anthony	2.01	21.7	8.00	3.06
Sangiovese	Sawmill	2.36	21.2	8.14	3.15
	Knapp	1.81	22.6	8.05	3.27
Cabernet Sauvignon	Fulkerson	1.38	20.6	10.30	3.18
	Sawmill	1.37	23.3	8.90	3.20
Cabernet franc	Knapp	1.49	21.0	7.97	3.27
	Lakewood	1.35	24.9	9.54	3.13

CHAPTER 3 SUPPLEMENTARY MATERIALS

Methods and Materials for Preliminary Observations–Pellets Formed Following Purified Seed Tannin Addition to Wines, Protein Identification and Compositional Analysis

SDS-PAGE visualization of proteins in wine pellets

To the re-dissolved pellets, 50 µL of sample loading buffer (24% glycerol, 0.1M Tris-HCl, 0.1% SDS, 2% β-mercaptoethanol, 0.02% Coomassie G-250, in filtered water (Millipore Corporation, Billerica, MA)) was added and gently mixed. Samples were heated for 5 min in a 95 °C water bath and immediately set on ice afterwards. Samples were then centrifuged briefly to recover droplets. Sample extracts were loaded at two different volumes (10 and 20 µL) into a 30 µL well on a Mini-PROTEAN TGX Precast 12% glycine gel (Bio-Rad Laboratories Inc., Hercules, CA), operating at 120V. Ten µL of a broad range molecular weight ladder (Precision Plus Protein Standard, Bio-Rad Laboratories Inc., Hercules, CA) was included on the gel. The proteins were fixed in the gel by a 50% methanol, 10% acetic acid solution for 1 h with agitation, then stained in 10% acetic acid, 0.025% Coomassie G-250 solution for 1 h with agitation, and then destained in three successive baths of 10% acetic acid for 1.5 h with agitation.

Protein Identification

Because all samples showed the same three bands via Coosmassie staining, only one of the samples (Corot noir, 20 µL loading) was selected for further characterization due to limited funds for analysis. This sample was selected because it displayed the best-defined bands. The in-gel digests performed at the Proteomics and Mass Spectrometry Facility at Cornell University were executed similar to the procedure described below. Protein analyses were performed by nanoLC-MS/MS using an UltiMate3000 MDLC (Dionex, Sunnyvale, CA) coupled to a hybrid triple quadrupole linear ion trap mass spectrometer (4000 QTrap, AB Sciex, Framingham, MA)

fitted with a MicroIonSpray II ion source. For identification, a representative in-gel digested sample (Corot noir, 20 µL loading) was reconstituted in 25 µL 2% acetonitrile (ACN)/0.5% formic acid (FA) and 10 µL was injected via autosampler onto a PepMap C18 trap column (5 µm, 100 µm, 5 mm, Dionex) with 0.1% FA at 20 µL/min for 3 min, then separated on a PepMap C18 RP nano column (3 µm, 75 µm x 15 cm, Dionex) using a 60 min linear gradient from 5% to 35% ACN in 0.1% FA at 300 nL/min, followed by a 3-min ramp to 95% ACN/0.1% FA and a 5-min hold at 95% ACN/0.1% FA. Information Dependent Acquisition (IDA) data was acquired using Analyst 1.4.2 software (AB Sciex) in positive ion enhanced mode by acquiring a MS survey scan from 375 – 1400 Da at 4000 amu/s followed by ER scans of the top three ions at 250 amu/s then summing two tandem MS (MS/MS) scans from 100 – 1600 Da at 4000 amu/s using Q0 trapping, fixed 20 ms trap filling time and rolling collision energy for up to three selected multiple-charge ions per cycle. The selected ions were then dynamically excluded for 60 s.

The acquired nanoLC-MS/MS IDA data was queried against the current *Vitis vinifera* reference database using the Mascot ver. 2.3 (Matrix Science Boston, MA) search engine. The search parameters specified a maximum of two trypsin miscleaves, fixed modification of carbamidomethyl-cysteine and variable modifications deamidation of asparagine/glutamine and oxidation of methionine.

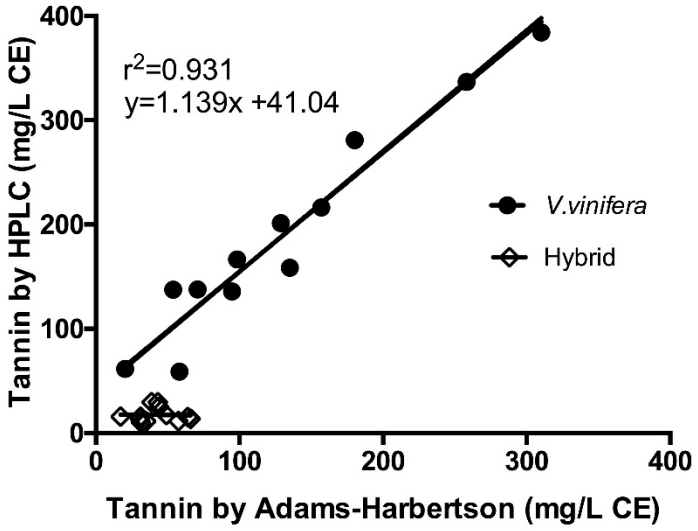
In-Gel Digest Protocol

For in-gel digestions – to each tube, gel pieces were washed with 500 µL filtered water, the sample was centrifuged at 8,000g for 30 s to pellet gel pieces, and excess water was removed following a 5 min wait period. Five hundred µL of a 50:50 solution of 100 mM ambic:ACN solution was added to the tube and vortexed. After 10 min, the gel pieces were pelleted by

centrifugation and excess liquid was removed. Five hundred µL of ACN was added and samples were mixed by vortexing. After 5 min, excess ACN was removed and gel pieces were dried in a Vacufuge™ for 20 min. Four hundred µL of 10 mM DTT was added to the dried pieces, and tubes were incubated in a 56 °C water bath for 45 min. Tubes were cooled to room temperature, 400 µL of 55 mM IAM was added, and samples were vortexed. Alkylation proceeded for 50 min in the dark, followed by another water washing step and ACN dehydration and drying cycle. Sequencing grade trypsin (20 µg) was rehydrated in 200 µL of trypsin rehydration buffer (50 mM acetic acid) in an ice bath, and further diluted to 0.002 µg/µL by addition of 50 mM ambic in 10% ACN (1176 µL) at 4 °C. The trypsin solution (100 µL) was added to each sample tube and the dried gel pieces allowed to rehydrate for 20 min. Additional 50 mM ambic (400 µL) was added to cover the rehydrated gel pieces, and samples incubated in a 30 °C water bath overnight for the trypsin digestion. Formic acid (5.1 µL) was then added to quench trypsin digestion.

For peptide fragment extraction, samples were vortexed, centrifuged, and the supernatant was removed and saved. ACN/H_2O (50:50, with 5% formic acid) was added to the gel pieces, incubated for 45 min, sonicated for 5 min, and supernatant removed and combined with the previous supernatant. This extraction step was repeated. Finally, 250 µL of 90% ACN with 5% formic acid was added, and samples were again centrifuged and supernatant collected. The pooled supernatants were dried in a Vacufuge™ before rehydrating for the relative quantitation of selected proteins using multiple reaction monitoring.

FIGURE S3.1Corrleation Between CT Quantification Methods



CT measured by the Adams-Harbertson protein precipitation assay vs. HPLC phloroglucinolysis for experimental wines produced from *V. vinifera* and interspecific hybrid grapes.

TABLE S3.1 Basic Juice Chemistry for the 2013 Grape Harvest

Culitvar	Location	Soluble Solids (° Brix)	Titratable Acidity (g/L)	pH	Berry Weight (g)
V. aestivalis	USDA Germplasm	19.2	25.80	3.34	0.94
V. cinerea	USDA Germplasm	21.3	22.17	3.11	0.29
V. riparia	USDA Germplasm	22.4	15.41	3.51	0.30
Baco noir	Lakewood Vineyards	19.0	11.23	3.32	1.25
Chancellor	Fulkerson Winery	17.3	11.14	3.23	1.75
DeChaunac	Knapp Winery	18.9	10.95	3.19	1.76
Maréchal Foch	Prejean Winery	22.7	11.01	3.42	1.15
Cabernet franc	Knapp Winery	21.4	7.02	3.45	1.67
Cabernet Sauvignon	Fulkerson Winery	20.5	9.89	3.41	1.38
Merlot	Prejean Winery	22.9	6.51	3.41	1.51
Pinot noir	Fulkerson Winery	21.2	9.10	3.40	1.60

TABLE S3.2 – Multiple Reaction Monitoring (MRM) Parameters for Protein Quantification by nanoLC-MS/MS

Targeted Protein (accession number)	MRM Targeted Peptides	M/Z	Z	Rention Time (min)
glucan endo-1,3-beta-glucosidase [Vitis vinifera] (gi|225441373)	282-TYNSNLIQHVK-292	658.90	2+	20.0
		439.70	3+	20.0
	323-HWGLFLPNK-331	556.4	2+	24.0
class IV chitinase precursor [Vitis vinifera] (gi|33329392)	225-AINGAVECNGGNTAAVNAR-243	930.50	2+	17.0
		620.40	3+	16.9
	249-DYCSQLGVSPGDNLTC-264	893.40	2+	30.9
VVTL1 precursor [Vitis vinifera] (gi|52617633)	155-APGGCNNPCTVFK-167	711.40	2+	21.3
	168-TNEYCCTDGPGSCGPTTYSK-187	1127.97	2+	18.4
peroxidase 4 [Vitis vinifera] (gi|223635590)	177-FQAQGLSTR-185	504.30	2+	17.2
	288-TFTSDFVAGMIK-299	666.90	2+	32.9
thaumatin-like protein [Vitis vinifera] (gi|225426795)	160-TTGGCNNPCTVFK-172	728.40	2+	19.9
	143-GISCTADIVGECPAALK-159	881.40	2+	30.3
Internal Standard Myoglobin [Equus burchelli]	33-LFTGHPETLEK-43	636.30	2+	20.4

TABLE S3.3 – Juice and Wine CT and Protein Quantities

Classification	Cultivar	Wine Tannin HPLC [1]	Skin Tannin [2]	Seed Tannin [2]	Total Tannin [2]	Tannin Precipitated After Addition [1]	Wine Protein [3]	Juice Protein [3]	Wine:Juice Protein Ratio [4]
Native *Vitis Spp.*	*V. aestivalis*	18.6 ± 4.1 e	0.137 ± 0.013 j	1.280 ± 0.049 defg	1.395 ± 0.036 efg	82.6 ±18.4 b	92.0 ± 19.2 bc	404.5 ± 50.5 b	22.7
	V.cinerea	28.9 ±1.0 de	0.380 ± 0.006 d	1.970 ± 0.028 bcd	2.350 ± 0.034 bcd	122.5 ± 3.4 a	118.3 ± 15.9 bc	146.4 ± 18.0 h	80.8
	V.riparia	30.2 ± 2.1 de	0.211 ± 0.020 f	1.580 ± 0.152 cde	1.791 ± 0.171 cde	130.7 ± 2.7 a	676.0 ± 65.1 a	1566.1 ± 99.6 a	43.2
	Average	**25.9 ± 20.5 B**	**0.162 ± 0.06 B**	**1.60 ± 0.15 B**	**1.85 ± 0.15 B**	**112.0 ± 5.6 A**	**295.5 ± 51.2 A**	**705.7 ±117.6 A**	**48.9**
Interspecific Hybrids	Baco noir	11.7 ±1.0 e	0.138 ± 0.013 i	1.150 ± 0.078 efg	1.288 ± 0.067 efg	51.7 ± 0.825 bcde	85.33 ± 17.5 bc	185.6 ± 17.6 f	46.0
	Chancellor	23.4 ±4.2 de	0.185 ± 0.003 g	0.794 ± 0.054 fg	0.979 ± 0.054 fg	56.0 ± 1.9 bcd	133.3 ± 19.2 b	171.7 ± 23.7 g	77.6
	DeChaunac	19.9 ± 3.4 de	0.161± 0.008 h	0.545 ± 0.012 g	0.706 ± 0.006 g	47.4 ±1.3 cde	37.3 ± 8.7 bc	41.7 ± 4.4 k	89.4
	Maréchal Foch	13.7 ± 1.2 e	0.112 ± 0.016 k	1.524 ± 0.063 cdef	1.626 ± 0.072 def	72.5 ± 2.6 bc	119.9 ± 26.4 bc	304.0 ±40.3 c	39.4
	Average	**17.4 ± 17.7 B**	**0.149 ± 0.05 B**	**1.00 ± 0.13 C**	**1.15 ± 0.13 C**	**57.0 ± 4.8 B**	**91.7 ± 44.4 B**	**175.8 ± 101.8 B**	**63.1**
V. vinifera	Cabernet franc	85.9 ±25.9 cd	0.553 ± 0.041 c	2.221 ± 0.336 abc	2.774 ± 0.345 b	22. 5 ± 1.2 e	36.6 ±11.8 bc	254.4 ± 4.23 d	14.4
	Cabernet Sauvignon	194.8 ±14.8 b	0.766 ± 0.047 b	1.772 ± 0.183 cde	2.538 ± 0.229 bc	35.1 ± 4.2 de	4.3 ±1.1 c	44.0 ± 4.0 j	9.8
	Merlot	334.0 ± 29.9 a	1.020 ± 0.051 a	2.556 ± 0.023 ab	3.576 ± 0.063 a	44.9 ± 2.9 cde	14.2 ± 3.2 bc	64.5 ± 14.9 i	22.0
	Pinot noir	144.0 ± 7.3 bc	0.300 ± 0.011 e	2.812 ± 0.240 a	3.112 ± 0.230 ab	33.0 ± 5.8 de	8.7 ±2.1 c	221.9 ± 12.1 e	3.9
	Average	**189.7 ± 17.7 A**	**0.660 ± 0.05 A**	**2.3 ± 0.13 A**	**3.0 ± 0.13 A**	**33.9 ± 4.8 C**	**16.0 ± 44.0 B**	**146.2 ±101.8 B**	**12.5**

[1] mg/L CE; [2] mg CE/g berries; [3] mg/L; [4] %

Similar letters within columns denote significant differences ($p<0.05$)

TABLE S3.4 – Relative Amounts of Quantified Proteins in Wines Prior to CT Addition

	Beta- glucanase	Class IV- Chitinase Precursor	VVTL1 Precursor	Thaumatin-like protein	Peroxidase-4
V. aestivalis	1.9	7.4	1.7	233	2.7
V. cinerea	3.9	34.1	1.2	452	39.8
V. riparia	100	100	100	100	100
Baco noir	20.9	178.6	77.1	686	8.2
Chancellor	97.8	1378.2	195.1	660	0.8
DeChaunac	13.5	509.5	66	189	0.4
Maréchal Foch	30.5	198	177	550	14.6
Cabernet franc	0.9	99.2	16.7	153.5	0.5
Cabernet Sauvignon	0.2	6.7	0.4	10.4	0.2
Merlot	0.1	0.3	4.2	106.4	3.4
Pinot noir	0.04	14.3	0.9	60.7	0.03

Values are normalized to *V. riparia* concentrations (=100)

TABLE S4.1 Basic Juice Chemistry for Grape Samples Harvested from NY and CA

Grape Variety	Location	pH	Soluble Solids (° Brix)	TA
Experiment 1				
Cabernet franc	CA	4.39	22.1	3.84
Cabernet Sauvignon	CA	4.16	21.4	4.33
Lemberger	CA	4.35	18.7	6.13
Dechaunac	CA	4.13	22.0	4.61
Maréchal Foch	CA	4.42	23.0	4.57
Regent	CA	4.42	26.5	3.50
Dornfelder	CA	3.99	20.9	3.20
Cabernet franc	NY	3.53	20.4	7.27
Cabernet Sauvignon	NY	3.56	21.2	7.77
Lemberger	NY	3.54	20.4	5.69
Dechaunac	NY	3.45	16.4	7.69
Maréchal Foch	NY	3.48	23.2	8.31
Rougeon	NY	3.25	13.7	11.99
Vincent	NY	3.30	13.2	11.62
Baco Noir	NY	3.34	17.8	14.40
Chancellor	NY	3.06	17.0	7.50
Experiment 2				
Maréchal Foch	NY	3.3	24.5	7.00
Lemberger	NY	3.3	22.3	8.30

Soluble solids as degrees brix, and titratable acidity (TA) in g/L

TABLE S4.2 CT in Lemberger Red Wines Before and After Experimental Juice Treatment, and Throughout the Duration of CT Extraction

Lemberger	*Before Treatment*	*After Treatment*	*Day 1*	*Day 2*	*Day 3*	*Day 4*	*Day 5*	*Day 6*	*Day 7*	*Day 8*	*Day 9*
Heating	N.D.	N.D.	N.D.	N.D.	103.8±39.3	124.2±36.6	151.0±59.0	168.5±53.3	187.0±49.9	213.7±94.4	231.4±77.1
Freezing	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	167.5±100.6	217.8±37.4	180.2±75.9	187.9±69.7	149.4±31.1
Tannin Addition	N.D.	236±52.8	103.1±10.7	129.7±23.9	199.3±39.7	177.8±20.5	274.8±144.5	326.3±145	289.6±92.7	293.6±48.6	282.3±85.2
Bentonite	N.D.	N.D.	N.D.	N.D.	N.D.	135.6±36.5	156.3±27.5	198.2±53.2	198.6±31.5	232.2±47.8	239.5±24.4
Control	N.D.	N.D.	N.D.	N.D.	N.D.	118.4±33.6	132.5±55.6	164.1±59.6	182.5±32.5	201.8±49.8	184.4±24.3

TABLE S4.3 CT in Maréchal Foch Wines Measured by HPLC-Phloroglucinolysis After 6 Months Bottle Storage

Treatments	Condensed Tannin (mg/L)
Heating	91.4±28.4
Freeze	74.6±14.4
Tannin Addition	107.6±2.8
Bentonite	77.5±0.7
Control	115.1±40.3

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